ABSTRACT

Title of Dissertation:	UNDERSTANDING THE REPRODUCTIVE BIOLOGY AND ENDOCRINOLOGY OF THE FEMALE RED DEEP-SEA CRAB, CHACEON QUINQUEDENS
	Shadaesha Renee Green, Doctor of Philosophy, 2022
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The red deep-sea crab, *Chaceon quinquedens*, a cold-water decapod found along the continental shelf and slope of the eastern United States has a limited amount of literature available describing its physiological processes. The present research aimed to advance the

current knowledge of the reproductive physiology of *C. quinquedens* females by investigating essential hormones regulating vitellogenesis and ovarian development.

In decapod crustaceans, members of the crustacean hyperglycemic hormone (CHH) superfamily control molting, growth, and reproduction, while reproduction and somatic growth are antagonistic. The red deep-sea crab with an extended intermolt period may adopt a similar approach to the blue crab, *Callinectes sapidus,* which utilizes the molt-inhibiting hormone (MIH) as a gonad-stimulating factor. Hence, a relationship between MIH and CHH levels and vitellogenin is examined, together with the potential role of other eyestalk neuropeptides in vitellogenesis using a transcriptomic analysis. Ten of the 28 eyestalk-neuropeptides found in the *de novo* assembly are differentially expressed between ovarian stages 1 and 3, suggesting their role in vitellogenesis.

The onset of vitellogenesis (synthesis of vitellogenin, the precursor of vitellin) initiates the ovarian development of all oviparous animals. Decapod crustaceans mainly utilize two tissues for vitellogenesis: hepatopancreas and ovary. The hepatopancreas of most crab species is the primary site for vitellogenesis, producing >99.9% of vitellogenin for ovarian development, while the ovary takes up vitellogenin subunits from the hemolymph. It is found using qPCR assay that the hepatopancreas of *C. quinquedens* also is the main site and provides >99.9% of vitellogenin. The following is then investigated on how the vitellogenin is cleaved into two subunits using a transcriptomic analysis. First, two transcripts are pieced together to obtain the putative 2,570 amino acid vitellogenin protein. Following three subtilisin-like endopeptidases are found in the *de novo* assembly, potentially cleaving vitellogenin into subunits: trypsin-like serine protease, furin, and proprotein convertase subtilisin/Kexin7.

Since the 1970s, the red deep-sea crab has been supporting a small fishing industry in the Atlantic Ocean, mainly harvesting adult males with >94 mm carapace width. However, a recent study reports that there is a reduction in the size of the males. To address the population structure of this species in the future, microsatellite markers are developed using MiSeq data combined with a bioinformatics pipeline. Over 37,000 microsatellites are identified, from which 122 markers are considered for initial PCR testing with a limited number of crabs. Overall, 14 novel, polymorphic microsatellite markers are developed.

In conclusion, the reproduction of female *C. quinquedens* is regulated by eyestalk neuropeptides. With this study, the reproductive role of MIH needs investigation in decapod crustaceans experiencing an extended intermolt stage. Novel microsatellite markers developed here will assist to study population structure and connectivity that will help towards the sustainable management of this species facing increasing fishing pressure.

UNDERSTANDING THE REPRODUCTIVE BIOLOGY AND ENDOCRINOLOGY OF THE FEMALE RED DEEP-SEA CRAB, CHACEON QUINQUEDENS

by

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Dissertation submitted to the Faculty of the Graduate School of the
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Dedication

I would like to dedicate this dissertation to the cherished memory of my mother, *Monae Reid-Tompkins*, Aunt *Tajuana Reid*, Grandmother, *Jessica Winslow-Reid*. While I have embarked on this journey without your physical presence, your encouragement for me to pursue my doctoral degree has been my ultimate motivation.

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(*Warning this may be the longest acknowledgments section ever, so go on and skip ahead to page vi if you don't care to read it…although that would be very rude of you)

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We finally made it y'all...

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List of Abbreviations

AK	Arginine Kinase
AKH	Adipokinetic Hormone
BP	Base Pair
BUSCO	Benchmarking Universal Single-Copy Orthologs
CCAP	Crustacean Cardioactive Peptide
cDNA	Complementary Deoxyribonucleic Acid
CFSH	Crustacean Female Sex Hormone
CHH	Crustacean Hyperglycemic Hormone
Chq	Chaceon quinquedens
CPRP	CHH Precursor-Related Peptide
DH	Diuretic Hormone
DNA	Deoxyribonucleic Acid
EH	Eclosion Hormone
GAIT	Gamma Interferon Inhibitor of Translation element
HC	Hudson Canyon
HPLC	High-Performance Liquid Chromatography
HWE	Hardy-Weinberg Equilibrium
kDa	Kilodalton
MAB	Mid-Atlantic Bight
MF	Methyl Farnesoate
MIH	Molt Inhibiting Hormone
MOIH	Mandibular Organ Inhibiting Hormone
mRNA	Messenger Ribonucleic Acid
NCBI	National Center for Biotechnology Information
NGS	Next-generation sequencing
NC	Norfolk Canyon
PCR	Polymerase Chain Reaction
qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic Acid
SE	Standard Error
SG	Sinus Gland
TOP	Terminal Oligopyrimidine Tracts
TPM	Transcripts Per Million
uORF	Upstream Open Reading Frame
UTR	Untranslated Region
V/GIH	Vitellogenin/Gonad-Inhibiting Hormone
VTG	Vitellogenin
XO	X-Organ

Chapter 1: General Introduction

1.1 Introduction to Crustaceans

Arthropoda is the largest phylum in the animal kingdom and contains the majority (~84%) of described species in the world. The phylum Arthropoda is a large group of invertebrates composed of insects, myriapods, arachnids, and crustaceans. They are found in every habitat on earth from aquatic to terrestrial environments (Ma et al., 2013; Thorp and Rogers, 2015). The growth of arthropod species is unique in that some have distinctive in that it is discontinuous, and animals are confined to a hard-shelled exoskeleton.

Within the phylum Arthropoda is the subphylum Crustacea which contains the class Malocrustacea. This class is comprised of a diverse group of invertebrates such as lobsters, shrimp, crayfish, prawns, and crabs. Growth in crustaceans is characterized by a series of molts (shedding of the exoskeleton) (Hartnoll, 1988). There is an intricate balance between the processes of growth and reproduction in all crustacean species.

In species such as the blue crab, *Callinectes sapidus* as the female matures, she undergoes a pubertal (also referred to as terminal molt) molt (van Engel, 1958). Other decapod species also exhibit this pattern such as *Libinia emarginata, Homarus gammarus, Maja squinado,* and *Chionoecetes opilio* (Gonzalez-Gurriarhn et al., 1995; Hinsch, 1972; Sampedro et al., 2003; Tamone et al., 2005). This molt is vital for reproductive activities to commence, starting with soft-shelled mating. In subsequent months the ovary matures while spermatophores are stored in her spermatheca until needed to fertilize eggs. A terminal molt also begins the reproductive process in the snow crab, *C. opilio*. Copulation in *C. opilio* females can happen when they are freshly molted (soft-shell), and they are also capable of mating with a hard shell (Hartnoll, 1969).

1.2 Neuropeptides as Reproductive Regulators

Reproduction is the most important physiological process for all life forms on earth. No matter the chosen molting-mating strategy all reproductive activities are stimulated and/or

inhibited by a cascade of neuroendocrine peptides. The eyestalk ganglia of crustaceans serve as the epicenter for the production, storage, and release of neuroendocrine hormones. Neurosecretory cells comprise the X-organ, which is the site of synthesis for neuropeptides. The neurohemal sinus gland is the location where neuropeptides are stored and secreted into the hemolymph. Together these two are termed the X-organ-sinus gland complex (XO-SG complex) and are connected to one another via an axonal tract which allows neuropeptides to travel from the site of synthesis to the place of storage and/or release.

Members of the crustacean hyperglycemic hormone (CHH) superfamily have been well studied and reviewed. Structurally, members of the CHH superfamily are similar to each other based on their conserved peptide sequences and three disulfide bridges (Böcking et al., 2002; Chen et al., 2020; Chung et al., 2010; Ohira, 2021). This family is subdivided into types I and II, which are distinguishable by the presence or absence of precursor peptides. Type I family members consist of CHH peptides that contain CHH precursor related peptide (CPRP), a signal peptide, and a mature CHH peptide (as well as the ion transport peptides-ITP in insects). The mature peptide is ~72 amino acids in length with an amidated C-terminus, and pyroglutamated N-terminus (in crabs, crayfish, and lobsters).

Type II family members are made up of the molt-inhibiting hormone (MIH), the vitellogenin/gonad-inhibiting hormone (V/GIH), and the mandibular organ-inhibiting hormone (MOIH). As implied the names of each peptide suggest, a variety of physiological functions are regulated via the type II family hormones including growth, metabolism, and reproduction. These prohormones contain a signal peptide and a preprohormone. The mature neuropeptides in this family are shorter in length since they only contain a signal peptide and preprohormone. Type II members also are lacking modifications at both the N- and C-termini and open ends

(Katayama et al., 2013; Ohira, 2021, 2016). The short N-terminal peptide present with a glycine residue at position 12 provides the structural difference between type I and 2 (Katayama and Nagasawa, 2004). Depending on the species, within these two subgroups, multiple peptide isoforms may be present for one or more of the members.

Reproduction is a key biological event in the life cycle of any organism. Molting and reproduction are two high energy-demanding processes, specifically in the life history of female crustaceans (Raviv et al., 2008). Within the class, Malacostraca are in the order Decapoda (or decapods) which includes lobsters, shrimp, prawns, crayfish, and crabs. In crabs, the timing of reproduction is influenced by a cascade of internal cues that control the complex mechanisms beneath molting and ovarian development. An organism's environment is also very instrumental in which reproductive strategy they exhibit. There have been many studies carried out exploring the antagonistic relationship between molting and reproduction in female crustaceans (Raviv et al., 2008).

Molting and reproduction in decapods such as crayfish, crabs, lobsters, prawns, and shrimp occur at two distinctive periods in their life history. The reproductive strategy of female crustaceans may also vary based on species, and/or environment (Raviv et al., 2006). It is important to understand the relationship between molting and reproduction due to their antagonistic behavior in many decapod species. In *C. sapidus*, females exhibit a pubertal terminal molt (Hines, 1988), where the female ceases to grow once, she is an adult.

1.2.1 Crustacean Hyperglycemic Hormone (CHH)

In recent years, the crustacean hyperglycemic hormone has been of special interest in the field of crustacean endocrinology due to its pleiotropic nature. The primary function of CHH in

crustaceans is the regulation of glucose metabolism. CHH is a hormone that is influenced by a variety of factors including stress, salinity, exercise, pressure, and temperature.

There is little information regarding the structure and functionality of CHH and its family members in deep-water crustaceans. The first CHH sequence of a cold-water species was the identification and functional investigation was from the Alaskan Tanner crab, *Chionoecetes bairdi* (Chung et al., 2009). The vent crab *Bythograea thermydronis* another deep-water crustacean and it resides in a much different environment (Toullec et al., 2002). Most recently, Chung et al., (2015) reported the CHH cDNA and deduced amino acid sequence for two snow crabs, *C. opilio* and *C. japonicus*. Analysis of the 5' and 3' untranslated regions of *Chionoecetes* species CHH sequences exhibit numerous regulatory motifs (i.e., uORFs, TOP, IRES, and GAIT) (Chung et al., 2015) that suggest a possible strong translational control of CHH in deeper water organism (Chung et al., 2015). It warrants further investigation.

CHHs have also been studied to understand their potential impact on molting and reproduction. In the American lobster, *Homarus americanus*, CHH-A & B were expressed in high levels during pre-vitellogenesis, while CHH-B (major form) was primarily responsible for the stimulation of oocyte maturation in females (de Kleijn et al., 1998). CHH in shrimp species serves the function of gonad-inhibiting hormone. However, in the blue crab, CHH did not show any influence on vitellogenesis. (Zmora et al., 2009b, 2009a).

1.2.3 Molt-Inhibiting Hormone (MIH)

In crustaceans, the molt-inhibiting hormone (MIH) works to suppress ecdysteroid, a molting hormone produced by the Y-organ. MIH levels are typically at their highest during the intermolt period, during which time circulating ecdysteroids are low (Chung and Webster, 2005, 2003; Keller and Schmid, 1979). The Y-organs (YO) are epithelial organs where steroid

hormones or ecdysteroids are synthesized and released into the hemolymph (Skinner, 1985). For the animal to gain an increase in size it must rid itself of its hard exoskeleton through the process of ecdysis, commonly referred to as molting. This highly coordinated event is brought about by an increase in the levels of ecdysteroids in the hemolymph that prepare the animal for ecdysis. Depending on the species, the YO may produce several active forms of circulating ecdysteroids that are present at different quantities at different life stages within the hemolymph. In the crabs, *C. sapidus* and *C. maenas*, ponasterone A (PoA) and 20-hydroxyecdysone (20-HE) are the major circulating ecdysteroids (Chung, 2010; Lachaise et al., 1989).

The typical molt cycle is separated into distinctive phases: (1) Proecdysis (or premolt) is the phase in which much of the old cuticle is absorbed and the new cuticle deposited; (2) Ecdysis (or molting) is brief and is the separation of the animal from its old exoskeleton to reveal its new exoskeleton; (3) Postecdysis (or postmolt) is the phase in which the newly formed cuticle at this phase consists of the new epicuticle and exocuticle at which point the animal is still soft and flexible; and (4) Intermolt, which is the longest stage in the molt cycle, where MIH levels are dominate and ecdysone levels are at a minimum.

Molt frequency slows as the crustaceans grow especially reaching adulthood. Some species stop molting and stay permanently in the intermolt phase by undergoing puberty-terminal molt upon reaching adulthood. In the blue crab, *C. sapidus* only females experience a puberty-terminal molt and remain at the intermolt stage, while males continue molting upon reaching the adult stage. In such cases, MIH plays a dual role: halting the animal at intermolt through maintaining low ecdysone levels in hemolymph and simultaneously stimulating vitellogenesis in hepatopancreas (Zmora et al., 2009b, 2009a).

Like other members of the CHH neuropeptide family, the action of MIH on other physiological processes is of recent interest, specifically in its connection to reproduction. In the blue crab, MIH simultaneously inhibits molting while stimulating vitellogenesis 1. In the sand shrimp, *Metapenaeus ensis*, MIH-B injections increased the vitellogenin mRNA and protein levels during vitellogenesis (Tiu and Chan, 2007). Likewise, in the Pacific white shrimp, *Litopenaeus vannamei*, a study investigating the impact of the two isoforms of MIH (1 & 2) on vitellogenesis found that MIH 2 stimulated the hepatopancreas production of vitellogenin and induced ovarian maturation 2. In addition to CHH superfamily members, there are other eyestalk-produced neuropeptides other than the members also involved in vitellogenesis (Nguyen V. T. et al., 2020; Ventura-López et al., 2017).

1.2.4 Mandibular Organ-Inhibiting Hormone (MOIH)

Mandibular organ-inhibiting hormone is produced and released from the XO-SG complex. MOIH is found only in Cancridae crabs (Chung et al., 2020; Wainwright et al., 1996). The primary role of MOIH is to inhibit the synthesis of methyl farnesoate (MF) in the mandibular organs. In *Cancer pagurus*, MOIH demonstrates continuous inhibition of methyl farnesoate and that its control on the mandibular organs fluctuates depending on the vitellogenic stage of the females (Tang et al., 1999; Wainwright et al., 1996). To date, MOIH is found in the species belonging to Cancridae (Chung et al., 2020). It is reported in the spider crabs that the structure of MOIH is the same as CHH (Liu et al., 1997), supporting the notion of CHH as a multifunctional hormone and suggesting that CHH may act as MOIH in other crustacean species.

Insect allatostatins have a similar function to crustacean MOIH, inhibiting methyl farnesoate synthesis by the mandibular organ (Liu et al., 2019; Wainwright et al., 1996). Therefore, the role of MF in the vitellogenesis of the hepatopancreas of decapod crustaceans

needs to be further studied, because it may play a role in ovarian vitellogenesis and maturation (Medesani et al., 2012; Reddy and Reddy, 2015).

1.2.5 Methyl Farnesoate

Methyl farnesoate is a sesquiterpene synthesized and released from the mandibular organs and is chemically similar to the insect juvenile hormone. Methyl farnesoate is known to be negatively influenced by MOIH (Liu and Laufer, 1986; Wainright et al., 1996). Injection of methyl farnesoate into *Procambarus clarkia* demonstrated a positive effect on oocyte growth (Rodríguez et al., 2002). In eyestalk ablated female spider crab injection of methyl farnesoate increases vitellogenin levels in the hemolymph (Vogel and Borst, 1989). Ovary maturation in *P. clarkii* is stimulated and enhanced by the addition of methyl farnesoate, *in vitro* (Laufer et al 1998). In *M. ensis* and *Charybdis feriatus*, farnesoic acid which is the precursor for methyl farnesoate incites the production of vitellogenin mRNA levels *in vitro* (Chan et al., 2005). Overall, MF is known as a gonad-stimulating factor in crustaceans. insect allatostatins, the function of which is equivalent to crustacean MOIH, inhibiting methyl farnesoate synthesis by the mandibular organ (Liu et al., 2019; Wainwright et al., 1996). Therefore, the role of methyl farnesoate in the vitellogenesis of the hepatopancreas of decapod crustaceans needs to be further studied (Medesani et al., 2012; Reddy and Reddy, 2015).

1.3 Ovarian Development and Vitellogenesis

Successful production of offspring of all ovipositors is contingent on ovarian growth and maturation. Ovarian development consists of two stages: primary and secondary vitellogenesis (Charniaux-cotton, 1985). Primary vitellogenesis is characterized by an increase in the number of

oocytes. Whereas secondary vitellogenesis is illustrated by the expansion in the size of the oocytes and the uptake of the yolk protein vitellin.

Vitellogenesis is an essential process for the successful completion of reproduction in all ovipositing animals. Vitellogenin the precursor of the main yolk protein vitellin is a large protein with just over 2500 amino acids in crustaceans. In decapods, the hepatopancreas and ovary are two major organs synthesizing the majority of vitellogenin (Jimenez-Gutierrez et al., 2019; Thongda et al., 2015). Vitellogenin produced in the hepatopancreas must be transported through the hemolymph to the oocytes where it will be absorbed via receptor mediated endocytosis (Meusy and Payen, 1988; Zmora et al., 2007). However, this is not before it experiences its first cleavage at an R-X-X-R motif. Subtilisin-like endopeptidases are responsible for the cleavage of these motifs found throughout the vitellogenin protein (Tseng et al., 2001).

Vitellogenesis is known to be regulated largely by the neuropeptide hormone(s) derived from the eyestalk ganglia (Chen et al., 2018, 2014; Tsutsui et al., 2013; Zmora et al., 2009b, 2009a). Such neuropeptides referred to as vitellogenesis-inhibiting/stimulating hormones (VI/SH) in shrimp and lobsters belong to the crustacean hyperglycemic hormone (CHH) superfamily (Chan et al. 2003). In crab species, the multifunctional hormone CHH has a role in vitellogenesis, whereas lobsters and shrimp utilize a specific structural form of VI/SH (Chen et al., 2014; Kluebsoongnoen et al., 2020; Vijayan et al., 2013).

Seasonal changes affect all physiological processes in aquatic animals including vitellogenesis. Warm water temperatures can decrease the time needed for species such as the blue crab, *Callinectes sapidus* (Thongda et al., 2015; Zmora et al., 2007), and the freshwater prawn, *Macrobrachium occidentale* (Hernández-Sandoval et al., 2018) to complete vitellogenesis. Cold waters slow down growth and increase the time needed for Chaceon females

to complete vitellogenesis and reproductive cycles (Biscoito et al., 2015; López-Abellán et al., 2002; Pezzuto and Sant' Ana, 2009).

Next-Generation Sequencing (NGS) has become an ideal tool used to investigate non-model organisms such as *C. quinquedens* that are not easily obtained and live in habitats that are difficult to mimic in a laboratory setting. Transcriptome generation from sequences produced by NGS technologies allows us to investigate specific physiological processes like reproduction. Transcriptomes produced from several crustaceans have aided in the study of reproductive activities in species such as *Scylla paramamosain* (Bao et al., 2015; Gao et al., 2014), *Eriochier sinensis* (He et al., 2012; X. Liu et al., 2019; Su et al., 2020), *Macrobrachium nipponense* (Jiang et al., 2019; Qiao et al., 2017; Zhang et al., 2021), *Portunus trituberculatus* (M. Liu et al., 2019; Meng et al., 2015; Wang et al., 2018; Yang et al., 2015), *Litopenaeus vannamei* (Wang et al., 2019), *Nephrops norvegicus* (Rotllant et al., 2018, 2017), *Sagmariasus verreauxi* (Ventura et al., 2014), and *Cherax quadricarinatus* (Nguyen et al., 2016).

1.4 Genetic Diversity

The ocean floor is the largest inhabited ecosystem on earth; however, the genetic composition of its many species has not been well studied (Taylor and Roterman, 2017). Some deep-sea crustaceans show a higher level of genetic variation compared to shallow-water species (Ramirez-Llodra et al., 2011, 2010; Taylor and Roterman, 2017). The employment of molecular markers in population genetic studies has revealed important information related to the biology, ecology, and evolution of many organisms. Molecular markers have been used to evaluate the genetic structure and connectivity of several ocean dwelling organisms that are easily assessable.

Next-generation sequencing technologies have increased the capabilities of researchers studying population genetics of almost any species. Non-model organisms living in areas that not

easily accessible and lacking genome data benefit greatly from the data produced by next-generation sequencing technologies and downstream analysis. Microsatellites or simple sequence repeats (SSRs) are tandem repeats of nucleotides (1-6 bp repeats) throughout the genome of an organism. Microsatellites are highly abundant within the genome of all species. Specifically, microsatellite markers are popular markers to employ in population genetic studies because of their highly polymorphic and codominant nature. As technologies advance the price and time it takes to obtain many microsatellites markers has decreased (Schuelke, 2000).

In crustaceans' studies using microsatellite markers to examine genetic diversity and population structure have increased in recent years. Development of a several microsatellite markers can be used for fisheries managers to understand genetic diversity and has been demonstrated by examining connectivity in several commercially important species i.e., *P. trituberculatus* (Xu and Liu, 2011), *C. opilio* (Puebla et al., 2003), *C. magister* (Toonen et al., 2004), *C. sapidus* (Steven et al. 2005), and *Scylla serrata* (Rumisha et al., 2017).

Microsatellites are often great markers for cross-species amplification and is a useful tool to help understand the population dynamics of related individuals. Markers developed for the freshwater crab *Sinopotamon yangtsekiense* and tested on seven related taxa proved to be specific to *Sinopotamon* (Yufang et al., 2009). Identification of the genetic structure of invasive species such as *C. maenas* using microsatellite markers is also important for managers of invaded territories to understand (Pascoal et al., 2009).

1.5 *Chaceon quinquedens*, Smith 1879

The Atlantic red deep-sea crab, *Chaceon quinquedens* inhabits the Atlantic Ocean coastal waters from Nova Scotia to the Gulf of Mexico at depths ranging from 200 - 1800 meters (Chute et al., 2008; Haefner and Musick, 1974; Steimle et al., 2001; Stevens and Guida, 2016; Weinberg

and Keith, 2003). It has supported a small commercial fishery along the eastern U.S. (from the Gulf of Maine to Cape Hatteras, NC) since the early 1970s (Serchuk, 1977; Steimle et al., 2001; Wahle et al., 2008; Wigley et al., 1975). Currently, the *C. quinquedens* fishing industry harvests adult males based on size, with carapace widths greater than 94 mm (Chute et al., 2008), while females are protected from harvesting. The reproductive biology of *C. quinquedens* with a life span of up to 15 years (Serchuk and Wigley, 1982), is largely unknown. Understanding physiological processes like vitellogenesis in this species is a necessity to increase the existing biological data that can potentially which can broaden our knowledge of this process in deep-sea crabs.

Morphological features of reproduction in females of the genus *Chaceon* have been studied for many years now (Attrill et al., 1991; Delgado and Defeo, 2004; Fernández-Vergaz et al., 2000; Ferreira et al., 2016; R Melville-Smith, 1988; Pinho et al., 2001; Tuset et al., 2011). Specifically, in *C. quinquedens* studies have looked at growth, fecundity, ovary maturation, mating, fishery status, and distribution.

Early studies on ovarian development provided a foundation for ovarian development (Haefner, 1977). Haefner (1977) described the ovary development stages. Ovary color and size were key parameters used to stage the ovary. Ovary stage by color is as follows, early *(*stage 1; colorless or white to beige*)*, intermediate *(*stage 2; yellow to light orange*)*, advanced (stage 3; bright orange to brown), mature (stage 4; red-orange to purple), and redeveloping (stage 5; beige). Recent work by Martinez (2018) examined the ovary by looking closely at oocyte maturation at each stage and corroborates the description of the ovary described by Haefner (1977). Additionally, laboratory studies examining the early life history of *C. quinquedens*

provided data on hatching and larval dispersal (Kelly et al., 1982; Perkins, 1973; Sulkin and van Heukelem, 1980; van Heukelem, 1983).

The size at sexual maturity (SM_{50}) of female red deep-sea crabs, in early studies, was found to range between 80-91 mm carapace width (Haefner, 1977). However, supplementary studies have observed ovigerous females as small as 61-79 mm carapace width (Elner et al., 1987; Stevens and Guida, 2016; Wigley et al., 1975). Recently, Martinez et al. (2020) describe a latitudinal trend in size at sexual maturity, where physiological SM_{50} increases from 61.2 mm to 70.8 mm carapace length as you go south from Hudson canyon to Norfolk Canyon, respectively. Along this same range, behavioral SM_{50} increased from 53.9 mm to 65.5 mm with decreasing latitudes from Hudson to Norfolk canyons (Martínez-Rivera et al., 2020).

Initial pre-copulatory mating behaviors for *C. quinquedens* begin as females approach ecdysis. Mating pairs are formed when large males form a protective cage (abdomen to abdomen) to guard the female until she molts (Elner et al., 1987). As the female emerges from her old shell the copulatory embrace begins, which can last on up to ~12 days (Elner et al., 1987). Elner et al. (1987) also observed the female's ability to mate with a hard shell like other cold-water species (Elner and Beninger, 1995; Raviv et al., 2008). Both spermatheca were filled, and there was no observation of a sperm plug or immediate extrusion of eggs in both soft and hard-shell females (Elner et al., 1987).

Duration of the reproductive cycle for some Geryonidae females occurs on an annual basis i.e., *Chaceon affinis*, *C. trispinosus*, *C. fenneri*, and *C. maritae* (Attrill et al., 1991; R. B Erdman et al., 1991; Hilário and Cunha, 2013; R. Melville-Smith, 1988; Pezzuto and Sant' Ana, 2009; Pinho et al., 2001). In addition to annual reproductive patterns, female *C. quinquedens* and other species within this genus have been suggested to exhibit a biennial reproductive cycle. This

pattern has been documented in *C. quinquedens, C. ramosae C. bicolor*, and *C. fenneri* (Robert B. Erdman et al., 1991; Haefner, 1977; Hilário and Cunha, 2013; Hines, 1988; Martinez-Rivera, 2018; Stevens and Guida, 2016).

Biennial reproductive cycles are normally observed in crabs living at depths where the water temperatures are very low (Comeau et al., 1999; Moriyasu and Lanteigne, 1998). *C. quinquedens* live at depths where temperatures can range between 5° and 8°C a proposed biennial reproductive cycle (Martinez-Rivera, 2018) could be due to intermolt duration as opposed to water temperature (Gerrior, 1981; Lux et al., 1982). Tagging studies of *C. quinquedens* showed that intermolt periods of large adults can last up to 7 years (Gerrior, 1981; Lux et al., 1982) with growth increments found to be <20 mm carapace width (Melville-Smith, 1989). A biennial reproductive cycle in *C. quinquedens* females could give them the opportunity to undergo at least three separate vitellogenesis events within a single molt cycle.

The fishery for *C. quinquedens* is currently managed as a single stock divided into three statistical areas (NEFMC, 2016). Many studies investigating *C. quinquedens* population structure, movement, and connectivity were published over a decade ago (Diehl and Biesiot, 1994; Lux et al., 1982; Wahle et al., 2008; Weinberg and Keith, 2003). Weinberg et al., (2003) examined the genetic diversity and subdivision of populations of this species from southern New England and the Gulf of Mexico regions using mitochondrial 16S rRNA markers. Their study found genetic subdivisions between *C. quinquedens* populations from New England and from the Gulf of Mexico using both 16S and ITS loci (Weinberg et al., 2003). The same 16S marker was used to examine members of the Chaceon genus. *C. fenneri* from New England and *C. affinis* from the Gulf had little to no genetic difference (Weinberg et al., 2003). Overall, both studies indicate a need for further research including larger sample sizes and additional populations.

1.6 Research Questions & Objectives

Past studies investigating the size, distribution, reproduction, stock status, and genetic connectivity of the red deep-sea crab currently make up the bulk of what we know about *C. quinquedens* (Biesiot and Perry, 1995; Elner et al., 1987; Haefner, 1978, 1977; Haefner and Musick, 1974; Wahle et al., 2008; Wigley et al., 1975). Most recently, studies on reproductive activities in this species have increased (Huang et al., 2020; Lawrence et al., 2017; Martínez-Rivera et al., 2020; Martínez-Rivera and Stevens, 2020; Stevens and Guida, 2016). Even with these foundational studies, there remains a deficiency within the published literature on hormonal regulation and vitellogenesis in *C. quinquedens* at the molecular level.

The research study outlined in this dissertation aims to further research on *C. quinquedens* female reproduction by investigating hormonal regulation, vitellogenesis, and ovarian development in the adult female. Knowledge of physiological processes such as vitellogenesis in C. *quinquedens* is necessary to understand how animals regulate ovarian development. a need for further research including larger sample sizes and additional populations. Sampling and collection of red deep-sea crabs and species like them residing at deeper depths present challenges to researchers, which makes understanding their genetic composition vital to the management of this species. In addition to studying reproduction, this research will also examine the population genetic structure of *C. quinquedens* within its fishery management areas will be conducted.

Chapter 2:

What eyestalk neuropeptides are found in female red deep-sea crabs at ovarian stages 1 (early-) and 3 (mid-vitellogenesis)?

a) Identification of CHH family neuropeptides including MIH and CHH present in the eyestalk of female red deep-sea crabs and their potential as reproductive regulators of ovarian development.
b) Mining of other peptides present in the eyestalk transcriptome.
c) Examine the differential expression of neuropeptides from the eyestalk transcriptome by comparing early and mid-vitellogenic stages.

Chapter 3:

Characterization of vitellogenin and cleaving enzymes expressed in the hepatopancreas of the red deep-sea crab during ovarian development?

d) Identify vitellogenin from the hepatopancreas and main its characteristics.
e) Identify cleaving enzymes such as subtilisin endopeptidases present.
f) Examine the differential expression of vitellogenin and cleaving enzymes from hepatopancreas transcriptome by comparing early and mid-vitellogenic stages (1 and 3, respectively) of female red deep-sea crabs.

Chapter 4:

What is the current state of the red deep-sea crab population structure?

g) Development of microsatellite markers.
h) Update the knowledge known about the genetic structure of red deep-sea crab populations for the fishery management areas in the New England & Mid-Atlantic.

References

Attrill, M.J., Hartnoll, R.G., Rice, A.L., 1991. Aspects of the biology of the deep-sea crab *Geryon trispinosus* from the porcupine seabight. Journal of the Marine Biological Association of the United Kingdom 71, 311–328. https://doi.org/10.1017/S0025315400051626

Bao, C., Yang, Y., Huang, H., Ye, H., 2015. Neuropeptides in the cerebral ganglia of the mud crab, *Scylla paramamosain*: transcriptomic analysis and expression profiles during vitellogenesis. Scientific Reports 5. https://doi.org/10.1038/srep17055

Biesiot, P.M., Perry, H.M., 1995. Biochemical composition of the deep-sea red crab Chaceon quinquedens (Geryonidae): organic reserves of developing embryos and adults. Marine Biology 124, 407–416. https://doi.org/10.1007/BF00363914

Biscoito, M., Freitas, M., Pajuelo, J.G., Triay-Portella, R., Santana, J.I., Costa, A.L., Delgado, J., González, J.A., 2015. Sex-structure, depth distribution, intermoult period and reproductive pattern of the deep-sea red crab *Chaceon affinis* (Brachyura, Geryonidae) in two populations in the north-eastern Atlantic. Deep-Sea Research Part I: Oceanographic Research Papers 95, 99–114. https://doi.org/10.1016/j.dsr.2014.10.010

Böcking, D., Dircksen, H., Keller, R., 2002. The Crustacean Neuropeptides of the CHH/MIH/GIH Family: Structures and Biological Activities., in: Wiese, K. (Ed.), The Crustacean Nervous System. Springer, NY, pp. 84–97.

Chan, S.M., Mak, A.S.C., Choi, C.L., Ma, T.H.T., Hui, J.H.L., Tiu, S.H.K., 2005. Vitellogenesis in the red crab, *Charybdis feriatus*: contributions from small vitellogenin transcripts (CfVg) and farnesoic acid stimulation of CfVg expression., in: Annals of the New York Academy of Sciences. New York Academy of Sciences, pp. 74–79. https://doi.org/10.1196/annals.1327.008

Charniaux-cotton, H., 1985. Vitellogenesis and Its Control in Malacostracan Crustacea1 duce alone . During the first stage o lished . Data concerning the endocrin logenesis but during the reproductive (Mathieu-Capderou , 1980) and in the Secondary folliculogenesis , which is proba 206, 197–206.

Chen, H.Y., Toullec, J.Y., Lee, C.Y., 2020. The Crustacean Hyperglycemic Hormone Superfamily: progress made in the past decade. Frontiers in Endocrinology. https://doi.org/10.3389/fendo.2020.578958

Chen, T., Ren, C., Jiang, X., Zhang, L., Li, H., Huang, W., Hu, C., 2018. Mechanisms for type-II vitellogenesis-inhibiting hormone suppression of vitellogenin transcription in shrimp hepatopancreas: Crosstalk of GC/cGMP pathway with different MAPK-dependent cascades. PLOS ONE 13. https://doi.org/10.1371/journal.pone.0194459

Chen, T., Zhang, L.-P., Wong, N.-K., Zhong, M., Ren, C.-H., Hu, C.-Q., 2014. Pacific white shrimp (<i>Litopenaeus vannamei<I/>) vitellogenesis-inhibiting hormone (VIH) Is predominantly expressed in the brain and negatively regulates hepatopancreatic vitellogenin (VTG) gene expression. Biology of Reproduction 90, 1–10. https://doi.org/10.1095/biolreprod.113.115030

Chung, J., Christie, A., Flynn, E., 2020. Molecular cloning of crustacean hyperglycemic hormone (CHH) family members (CHH, molt-inhibiting hormone and mandibular organ-inhibiting hormone) and their expression levels in the Jonah crab, *Cancer borealis*. General and Comparative Endocrinology 295, 113522. https://doi.org/10.1016/j.ygcen.2020.113522

Chung, J.S., 2010. Hemolymph ecdysteroids during the last three molt cycles of the blue crab, *Callinectes sapidus*: quantitative and qualitative analyses and regulation. Archives of Insect Biochemistry and Physiology 73, 1–13. https://doi.org/10.1002/arch.20327

Chung, J.S., Ahn, I.S., Yu, O.H., Kim, D.S., 2015. Crustacean hyperglycemic hormones of two cold water crab species, *Chionoecetes opilio* and *C. japonicus*: isolation of cDNA sequences and localization of CHH neuropeptide in eyestalk ganglia. General and Comparative Endocrinology 214, 177–185. https://doi.org/10.1016/j.ygcen.2014.08.017

Chung, J.S., Bembe, S., Tamone, S., Andrews, E., Thomas, H., 2009. Molecular cloning of the crustacean hyperglycemic hormone (CHH) precursor from the x-organ and the identification of the neuropeptide from sinus gland of the Alaskan Tanner crab, *Chionoecetes bairdi*. General and Comparative Endocrinology 162, 129–133. https://doi.org/10.1016/j.ygcen.2009.03.012

Chung, J.S., Webster, S.G., 2005. Dynamics of in vivo release of molt-inhibiting hormone and crustacean hyperglycemic hormone in the shore crab, *carcinus maenas*. Endocrinology 146, 5545–5551. https://doi.org/10.1210/en.2005-0859

Chung, J.S., Webster, S.G., 2003. Moult cycle-related changes in biological activity of moult-inhibiting hormone (MIH) and crustacean hyperglycaemic hormone (CHH) in the crab,

Carcinus maenas: from target to transcript. European Journal of Biochemistry 270, 3280–3288. https://doi.org/10.1046/j.1432-1033.2003.03720.x

Chung, J.S., Zmora, N., Katayama, H., Tsutsui, N., 2010. Crustacean hyperglycemic hormone (CHH) neuropeptidesfamily: Functions, titer, and binding to target tissues. General and Comparative Endocrinology 166, 447–454. https://doi.org/10.1016/j.ygcen.2009.12.011

Chute, A., Jacobson, L., Rago, P., MacCall, A., 2008. Deep Sea red crab. Northeast Data Poor Stocks Working Group Meeting 1–18.

Comeau, M., Starr, M., Conan, G.Y., Robichaud, G., Therriault, J.-C., 1999. Fecundity and duration of egg incubation for multiparous female snow crabs (*Chionoecetes opilio*) in the fjord of Bonne Bay, Newfoundland. Canadian Journal of Fisheries and Aquatic Sciences 56, 1088–1095. https://doi.org/10.1139/f99-044

de Kleijn, D.P.V., Janssen, K.P.C., Waddy, S.L., Hegeman, R., Lai, W.Y., Martens, G.J.M., van Herp, F., 1998. Expression of the crustacean hyperglycaemic hormones and the gonad-inhibiting hormone during the reproductive cycle of the female American lobster Homarus americanus. Journal of Endocrinology 156, 291–298. https://doi.org/10.1677/joe.0.1560291

Delgado, E., Defeo, O., 2004. Sexual maturity in females of deep-sea red crab *Chaceon notialis* (Brachyura, Geryonidae) in the southwestern Atlantic Ocean. Invertebrate Reproduction and Development 46, 55–62.

Diehl, W.J., Biesiot, P.M., 1994. Relationships between multilocus heterozygosity and morphometric indices in a population of the deep-sea red crab *Chaceon quinquedens* (Smith). Journal of Experimental Marine Biology and Ecology 182, 237–250. https://doi.org/10.1016/0022-0981(94)90054-X

Elner, R.W., Beninger, P.G., 1995. Multiple reproductive strategies in snow crab, Chionoecetes opilio: Physiological pathways and behavioral plasticity. Journal of Experimental Marine Biology and Ecology 193, 93–112. https://doi.org/10.1016/0022-0981(95)00112-3

Elner, R.W., Koshio, S., Hurley, G.V., 1987. Mating behavior of the deep-sea red crab, *Geryon quinquedens* Smith (Decapoda, Brachyura, Geryonidae). Crustaceana 52, 194–201.

Erdman, R. B, Blake, N.J., Lockhart, F.D., Lindberg, W.J., Perry, H.M., Waller, R.S., 1991. Comparative reproduction of the deep-sea crabs *Chaceon fenneri* and *C. quinquedens* (Brachyura: Geryonidae) from the northeast Gulf of Mexico. Invertebrate reproduction & development 19, 175–184.

Erdman, Robert B., Blake, N.J., Torres, J.J., 1991. Oxygen consumption of the deep-sea crabs *Chaceon fenneri* and *C. quinquedens* (Brachyura: Geryonidae). Comparative Biochemistry and Physiology -- Part A: Physiology 99, 383–385. https://doi.org/10.1016/0300-9629(91)90020-D

Fernández-Vergaz, V., López Abellán, L.J., Balguerías, E., 2000. Morphometric, functional and sexual maturity of the deep-sea red crab *Chaceon affinis* inhabiting Canary Island waters: chronology of maturation. Marine Ecology Progress Series 204, 169–178. https://doi.org/10.3354/meps204169

Ferreira, R.C.P., Nunes, D.M., Shinozaki-Mendes, R.A., Pires, A.M.A., Hazin, F.H.V., 2016. First record and preliminary information on the biology of the deep-sea African crab, *Chaceon gordonae* (Ingle, 1985) (Brachyura: Geryonidae) in Saint Peter and Saint Paul Archipelago, Brazil. Latin American Journal of Aquatic Research 44, 392–400. https://doi.org/10.3856/vol44-issue2-fulltext-20

Gao, J., Wang, X., Zou, Z., Jia, X., Wang, Y., Zhang, Z., 2014. Transcriptome analysis of the differences in gene expression between testis and ovary in green mud crab (*Scylla paramamosain*). BMC Genomics 15. https://doi.org/10.1186/1471-2164-15-585

Gerrior, P., 1981. The distribution and effects of fishing on the deep-sea red crab, *Geryon quinquedens* Smith, off southern New England. North Dartmouth, MA.

Gonzalez-Gurriarhn, E., Freire, J., Parapar, J., Sampedro, M.P., Urcera, M., 1995. Growth at moult and moulting seasonality of the spider crab, *Maja squinado* (Herbst) (Decapoda: Majidae) in experimental conditions: implications for juvenile life history. Journal of Experimental Marine Biology and Ecology 189, 183–203.

Haefner, P., 1978. Seasonal aspects of the biology, distribution and relative abundance of the deep-sea red crab *Geryon quinquedens* Smith, in the vicinity of the Norfolk Canyon, Western North Atlantic. Proceedings of the National Shellfisheries Association 68, 49–62.

Haefner, P.A., 1977. Reproductive biology of the female Deep-Sea Red Crab, *Geryon quinquedens*, from the Chesapeake Bight. Fishery Bulletin 75, 91–102.

Haefner, P.A., Musick, J.A., 1974. Observations on distribution and abundance of red crabs in Norfolk Canyon and adjacent continental slope. Marine Fisheries Review 36, 31–34.

Hartnoll, R.G., 1988. Growth and molting., in: Burggren, W.W., McMahon, B.R. (Eds.), Biology of Land Crabs. Cambridge University Press.

He, L., Wang, Q., Jin, X., Wang, Ying, Chen, L., Liu, L., Wang, Yang, 2012. Transcriptome profiling of testis during sexual maturation stages in *Eriocheir sinensis* using Illumina sequencing. PLoS ONE 7. https://doi.org/10.1371/journal.pone.0033735

Hernández-Sandoval, P., Díaz-Herrera, F., Díaz-Gaxiola, J.M., Martínez-Valenzuela, C., García-Guerrero, M., 2018. Effect of temperature on growth, survival, thermal behavior, and critical thermal maximum in the juveniles of *Macrobrachium occidentale* (Holthuis, 1950) (Decapoda: Caridea: Palaemonidae) from Mexico. Journal of Crustacean Biology 38, 483–488. https://doi.org/10.1093/jcbiol/ruy024

Hilário, A., Cunha, M.R., 2013. Notes on a mating event of the deep-sea crab *Chaceon affinis* in the Gorringe Bank (NE Atlantic). Deep-Sea Research Part II: Topical Studies in Oceanography 92, 58–62. https://doi.org/10.1016/j.dsr2.2013.01.021

Hines, A.H., 1988. Fecundity and Reproductive Output in Two Species of Deep-Sea Crabs, Geryon fenneri and G. quinquedens (Decapoda: Brachyura), Source: Journal of Crustacean Biology.

Hinsch, G.W., 1972. Some factors controlling reproduction in the spider crab, *Libinia emarginata*. The Biological bulletin 143, 358–366. https://doi.org/10.2307/1540059

Huang, X., Green, S., Sook Chung, J., 2020. The presence of an insulin-like peptide-binding protein (ILPBP) in the ovary and its involvement in the ovarian development of the red deep-sea crab, *Chaceon quinquedens*. General and Comparative Endocrinology 301, 113653. https://doi.org/10.1016/j.ygcen.2020.113653

Jiang, Q., Min, Y., Yang, H., Wan, W., Zhang, X., 2019. De novo transcriptome analysis of eyestalk reveals ovarian maturation related genes in *Macrobrachium rosenbergii*. Aquaculture 505, 280–288. https://doi.org/10.1016/j.aquaculture.2019.02.073

Jimenez-Gutierrez, S., Cadena-Caballero, C.E., Barrios-Hernandez, C., Perez-Gonzalez, R., Martinez-Perez, F., Jimenez-Gutierrez, L.R., 2019. Crustacean vitellogenin: A systematic and experimental analysis of their genes, genomes, mRNAs and proteins; and perspective to Next Generation Sequencing. Crustaceana 92, 1169–1205. https://doi.org/10.1163/15685403-00003930

Katayama, H., Nagasawa, H., 2004. Effect of a Glycine Residue Insertion into Crustacean Hyperglycemic Hormone on Hormonal Activity. Zoological Science 21, 1121–1124. https://doi.org/10.2108/zsj.21.1121

Katayama, H., Ohira, T., Nagasawa, H., 2013. Crustacean Peptide Hormones: Structure, Gene Expression and Function. Aqua-BioScience Monographs 6, 49–90. https://doi.org/10.5047/absm.2013.00602.0049

Keller, R., Schmid, E., 1979. In vitro secretion of ecdysteroids by Y-organs and lack of secretion by mandibular organs of the crayfish following molt induction. Journal of Comparative Physiology B 130, 347–353. https://doi.org/10.1007/BF00689853

Kelly, P., Sulkin, S.D., van Heukelem, W.F., 1982. A Dispersal Model for Larvae of the Deep Sea Red Crab *Geryon quinquedens* Based upon Behavioral Regulation of Vertical Migration in the Hatching Stage*. Marine Biology 72, 35–43.

Kluebsoongnoen, J., Panyim, S., Udomkit, A., 2020. Regulation of vitellogenin gene expression under the negative modulator, gonad-inhibiting hormone in *Penaeus monodon*. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 243, 110682.

Lachaise, F., Carpentier, G., Sommé, G., Colardeau, J., Beydon, P., 1989. Ecdysteroid synthesis by crab Y-organs. Journal of Experimental Zoology 252, 283–292. https://doi.org/10.1002/JEZ.1402520311

Lawrence, A., Green, S., Chung, J.S., 2017. Isolation and tissue distribution of an insulin-like androgenic gland hormone (IAG) of the male red deep-sea crab, *Chaceon quinquedens*. Marine Drugs 15. https://doi.org/10.3390/md15080241

Liu, A., Liu, F., Shi, W., Huang, H., Wang, G., Ye, H., 2019. C-Type allatostatin and its putative receptor from the mud crab serve an inhibitory role in ovarian development. Journal of Experimental Biology 222. https://doi.org/10.1242/jeb.207985

Liu, L., Laufer, H., Gogarten, P.J., Wang, M., 1997. cDNA cloning of a mandibular organ inhibiting hormone from the spider crab Libinia emarginata. Invertebrate Neuroscience 3, 199–204. https://doi.org/10.1007/BF02480375

Liu, M., Pan, J., Dong, Z., Cheng, Y., Gong, J., Wu, X., 2019. Comparative transcriptome reveals the potential modulation mechanisms of estradiol affecting ovarian development of female *Portunus trituberculatus*. PLoS ONE 14, 1–21. https://doi.org/10.1371/journal.pone.0226698

Liu, X., Ma, K.Y., Liu, Z.Q., Feng, J. bin, Ye, B.Q., Qiu, G.F., 2019. Transcriptome analysis of the brain of the Chinese mitten crab, *Eriocheir sinensis*, for neuropeptide abundance profiles

during ovarian development. Animal Reproduction Science 201, 63–70. https://doi.org/10.1016/j.anireprosci.2018.12.010

López-Abellán, L.J., Balguerías, E., Fernández-Vergaz, V., 2002. Life history characteristics of the deep-sea crab *Chaceon affinis* population off Tenerife (Canary Islands). Fisheries Research 58, 231–239. https://doi.org/10.1016/S0165-7836(01)00384-8

Lux, F.E., Ganz, A.R., Rathjen, W.F., 1982. Marking studies on the red crab *Geryon quinquedens* Smith off southern New England. Journal of Shellfish Research 2, 71–80.

Ma, W., Vavre, F., Beukeboom, L., 2013. Manipulation of arthropod sex determination by endosymbionts: Diversity and Molecular Mechanisms. Sexual Development 8, 59–73.

Martinez-Rivera, S., 2018. Reproductive biology of the female red deep-sea crab, *Chaceon quinquedens* (Smith, 1879), in the Mid-Atlantic Bight.

Martínez-Rivera, S., Long, W.C., Stevens, B.G., 2020. Physiological and behavioral sexual maturity of female red deep-sea crabs *Chaceon quinquedens* (Smith, 1879) (Decapoda: Brachyura: Geryonidae) in the Mid-Atlantic Bight. Journal of Crustacean Biology 40, 330–340. https://doi.org/10.1093/JCBIOL/RUAA007

Martínez-Rivera, S., Stevens, B.G., 2020. Embryonic development and fecundity of the red deep-sea crab *Chaceon quinquedens* (Smith, 1879) (Decapoda: Brachyura: Geryonidae) in the Mid-Atlantic Bight determined by image analysis. Journal of Crustacean Biology 40, 230–236. https://doi.org/10.1093/JCBIOL/RUAA017

Medesani, D.A., Ferré, L.E., Grodzielski, M., Rodríguez, E.M., 2012. *In vitro* effect of methyl farnesoate on the vitellogenin content of ovary and hepatopancreas, in the crayfish *Cherax quadricarinatus*. Invertebrate Reproduction & Development 56. https://doi.org/10.1080/07924259.2011.582691

Melville-Smith, R., 1989. A growth model for the deep-sea red crab (*Geryon maritae* off South West Africa/Namibia (Decapoda, Brachyura). Crustaceana 56, 279–292.

Melville-Smith, R, 1988. The Commercial Fishery for and Population Dynamics of Red Crab Geryon maritae off Southwest Africa, 1976-1986. South African Journal of Marine Sciences 5, 79–95.

Melville-Smith, R., 1988. The commercial fishery for and population dynamics of the red crab <I>Geryon maritae</I> off South West Africa, 1976-1986. South African Journal of Marine Sciences 5, 79–95.

Meng, X.L., Liu, P., Jia, F.L., Li, J., Gao, B.Q., 2015. De novo transcriptome analysis of *Portunus trituberculatus* ovary and testis by RNA-Seq: Identification of genes involved in gonadal development. PLoS ONE 10. https://doi.org/10.1371/journal.pone.0128659

Meusy, J.-J., Payen, G.G., 1988. Female reproduction in Malacostracan crustacea regulation. Zoological Science 5, 217–265.

Moriyasu, M., Lanteigne, C., 1998. Embryo development and reproductive cycle in the snow crab, *Chionoecetes opilio* (Crustacea: Majidae), in the southern Gulf of St. Lawrence, Canada. Canadian Journal of Zoology 76, 2040–2048.

Nguyen, T.V., Cummins, S.F., Elizur, A., Ventura, T., 2016. Transcriptomic characterization and curation of candidate neuropeptides regulating reproduction in the eyestalk ganglia of the Australian crayfish, *Cherax quadricarinatus*. Scientific Reports 6, 1–19. https://doi.org/10.1038/srep38658

Nguyen V. T., Ryan, L.W., Nocillado, J., le Groumellec, M., Elizur, A., Ventura, T., 2020. Transcriptomic changes across vitellogenesis in the black tiger prawn (*Penaeus monodon*), neuropeptides and G protein-coupled receptors repertoire curation. General and Comparative Endocrinology 298, 113585. https://doi.org/10.1016/j.ygcen.2020.113585

Ohira, T., 2021. Chapter 76 - Crustacean hyperglycemic hormone, in: Ando, H., Ukena, K., Nagata, S. (Eds.), Handbook of Hormones. Elsevier, pp. 731–733. https://doi.org/10.1016/B978-0-12-820649-2.00196-0

Ohira, T., 2016. Crustacean Hyperglycemic Hormone. Handbook of Hormones 403–404, e53-1-e53-1. https://doi.org/10.1016/B978-0-12-801028-0.00422-0

Pascoal, S., Creer, S., Taylor, M.I., Queiroga, H., Carvalho, G., Mendo, S., 2009. Development and application of microsatellites in *Carcinus maenas*: genetic differentiation between northern and central Portuguese populations. PLoS ONE 4. https://doi.org/10.1371/journal.pone.0007268

Perkins, H.C., 1973. The larval stages of the deep sea red craab, *Geryon quinquedens* Smith, reared under laboratory conditions (Decapoda: Brachyrhyncha). Fisheries Bulletin 71, 69–82.

Pezzuto, P.R., Sant' Ana, R., 2009. Sexual maturity of the deep-sea royal crab *Chaceon ramosae* Manning, Tavares & Albuquerque, 1989 (Brachyura: Geryonidae) in southern Brazil. Latin American Journal of Aquatic Research 37, 285–296. https://doi.org/10.3856/vol37-issue3-fulltext-2

Pinho, M.R., Gonçalves, J.M., Martins, H.R., Menezes, G.M., 2001. Some aspects of the biology of the deep-water crab, *Chaceon affinis* (Milne-Edwards and Bouvier, 1894) off the Azores. Fisheries Research 51, 283–295.

Puebla, O., Parent, É., Sévigny, J.M., 2003. New microsatellite markers for the snow crab Chionoecetes opilio (Brachyura: Majidae). Molecular Ecology Notes 3, 644–646. https://doi.org/10.1046/j.1471-8286.2003.00542.x

Qiao, H., Fu, H., Xiong, Y., Jiang, S., Zhang, W., Sun, S., Jin, S., Gong, Y., Wang, Y., Shan, D., Li, F., Wu, Y., 2017. Molecular insights into reproduction regulation of female Oriental River prawns *Macrobrachium nipponense* through comparative transcriptomic analysis. Scientific Reports 7, 1–11. https://doi.org/10.1038/s41598-017-10439-2

Ramirez-Llodra, E., Brandt, A., Danovaro, R., de Mol, B., Escobar, E., German, C.R., Levin, L.A., Martinez Arbizu, P., Menot, L., Buhl-Mortensen, P., Narayanaswamy, B.E., Smith, C.R., Tittensor, D.P., Tyler, P.A., Vanreusel, A., Vecchione, M., 2010. Deep, diverse and definitely different: Unique attributes of the world's largest ecosystem. Biogeosciences 7, 2851–2899. https://doi.org/10.5194/bg-7-2851-2010

Ramirez-Llodra, E., Tyler, P.A., Baker, M.C., Bergstad, O.A., Clark, M.R., Escobar, E., Levin, L.A., Menot, L., Rowden, A.A., Smith, C.R., van Dover, C.L., 2011. Man and the last great wilderness: Human impact on the deep sea. PLoS ONE 6. https://doi.org/10.1371/journal.pone.0022588

Raviv, S., Parnes, S., Sagi, A., 2008. Coordination of Reproduction and Molt in Decapods. Reproductive Biology of Crustaceans 365–390. https://doi.org/10.1201/9781439843345-c9

Raviv, S., Parnes, S., Segall, C., Davis, C., Sagi, A., 2006. Complete sequence of *Litopenaeus vannamei* (Crustacea: Decapoda) vitellogenin cDNA and its expression in endocrinologically induced sub-adult females. General and Comparative Endocrinology 145, 39–50. https://doi.org/10.1016/j.ygcen.2005.06.009

Reddy, B.S., Reddy, P.R., 2015. Methyl farnesoate induced ovarian vitellogenin syntehsis in freshwater rice field crab *Oziothelphusa senex senex* Fabricious. Journal of Infertility and Reproductive Biology 3, 237–244.

Rodríguez, E.M., López Greco, L.S., Medesani, D.A., Laufer, H., Fingerman, M., 2002. Effect of methyl farnesoate, alone and in combination with other hormones, on ovarian growth of the

red swamp crayfish, Procambarus clarkii, during vitellogenesis. General and Comparative Endocrinology 125, 34–40. https://doi.org/10.1006/gcen.2001.7724

Rotllant, G., Nguyen, T.V., Hurwood, D., Sbragaglia, V., Ventura, T., Company, J.B., Joly, S., Elizur, A., Mather, P.B., 2018. Evaluation of genes involved in Norway lobster (*Nephrops norvegicus*) female sexual maturation using transcriptomic analysis. Hydrobiologia 825, 137–158. https://doi.org/10.1007/s10750-018-3521-3

Rotllant, G., Nguyen, T.V., Sbragaglia, V., Rahi, L., Dudley, K.J., Hurwood, D., Ventura, T., Company, J.B., Chand, V., Aguzzi, J., Mather, P.B., 2017. Sex and tissue specific gene expression patterns identified following de novo transcriptomic analysis of the Norway lobster, *Nephrops norvegicus*. BMC Genomics 18, 1–14. https://doi.org/10.1186/s12864-017-3981-2

Rumisha, C., Huyghe, F., Rapanoel, D., Mascaux, N., Kochzius, M., 2017. Genetic diversity and connectivity in the East African giant mud crab *Scylla serrata*: Implications for fisheries management. PLoS ONE 12. https://doi.org/10.1371/journal.pone.0186817

Sampedro, M.P., González-Gurriarán, E., Freire, J., 2003. Moult cycle and growth of *Maja squinado* (Decapoda: Majidae) in coastal habitats of Galicia, north-west Spain. Journal of the Marine Biological Association of the United Kingdom 83, 995–1005. https://doi.org/10.1017/S0025315403008208h

Schuelke, M., 2000. An economic method for the fluorescent labeling of PCR fragments. Nature Biotechnology 18, 233–234.

Serchuk, F.M., 1977. Assessment of red crab (*Geryon quinquedens*) populations in the northwest Atlantic. Northeast Fisheries Science Center Ref. Doc. 77, 1–15.

Serchuk, F.M., Wigley, R.L., 1982. Deep-sea red crab, *Geryon quinquedens*. Fish Distribution 15, 125–129.

Skinner, D.M., 1985. Interacting Factors in the Control of the Crustacean Molt Cycle1, Amer. Zool.

Steimle, F.W., Zetlin, C.A., Chang, S., 2001. Red deepsea crab, *Chaceon (Geryon) quinquedens*, life history and habitat characteristics., NOAA Tech. Memo. NMFS-NE-163.

Stevens, B.G., Guida, V., 2016. Depth and temperature distribution, morphometrics, and sex ratios of red deepsea crab (*Chaceon quinquedens*) at 4 sampling sites in the Mid-Atlantic Bight. Fishery Bulletin 114, 343–359. https://doi.org/10.7755/FB.114.3.7

Su, Y., Guo, Q., Gong, J., Cheng, Y., Wu, X., 2020. Functional expression patterns of four ecdysteroid receptor isoforms indicate their different functions during vitellogenesis of Chinese mitten crab, *Eriocheir sinensis*. Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology 248. https://doi.org/10.1016/j.cbpa.2020.110754

Sulkin, S.D., van Heukelem, W.F., 1980. Ecological and evolutionary significance of nutritional flexibility in planktotrophic larvae of the deep sea red crab *Geryon quinquedens* and the stone crab *Menippe mercenaria*. Marine Ecology - Progress Series 2, 91–95.

Tamone, S.L., Adams, M.M., Dutton, J.M., 2005. Effect of eyestalk-ablation on circulating ecdysteroids in hemolymph of Snow Crabs, *Chionoecetes opilio*: physiological evidence for a terminal molt. Integrative and Comparative Biology 45, 166–171.

Tang, C., Lu, W., Wainwright, G., Webster, S.G., Rees, H.H., Turner, P.C., 1999. Molecular characterization and expression of mandibular organ-inhibiting hormone, a recently discovered neuropeptide involved in the regulation of growth and reproduction in the crab *Cancer pagurus*. Biochem. J 343, 355–360.

Taylor, M.L., Roterman, C.N., 2017. Invertebrate population genetics across Earth's largest habitat: the deep-sea floor. Molecular Ecology 26, 4872–4896. https://doi.org/10.1111/mec.14237

Thongda, W., Chung, J.S., Tsutsui, N., Zmora, N., Katenta, A., 2015. Seasonal variations in reproductive activity of the blue crab, *Callinectes sapidus*: Vitellogenin expression and levels of vitellogenin in the hemolymph during ovarian development. Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology 179, 35–43. https://doi.org/10.1016/j.cbpa.2014.08.019

Thorp, J.H., Rogers, D.C., 2015. Introduction to the Phylum Arthropoda., in: Thorp, J.H., Rogers, D.C. (Eds.), Thorp and Covich's Freshwater Invertebrates. Elsevier, Elsevier, pp. 591–597. https://doi.org/10.1016/B978-0-12-385026-3.00024-3

Tiu, S.H.K., Chan, S.M., 2007. The use of recombinant protein and RNA interference approaches to study the reproductive functions of a gonad-stimulating hormone from the shrimp *Metapenaeus ensis*. FEBS Journal 274, 4385–4395. https://doi.org/10.1111/j.1742-4658.2007.05968.x

Toonen, R.J., Locke, M., Grosberg, R., 2004. Isolation and characterization of polymorphic microsatellite loci from the Dungeness crab Cancer magister. Molecular Ecology Notes 4, 30–32. https://doi.org/10.1046/j.1471-8286.2003.00562.x

Toullec, J.Y., Vinh, J., le Caer, J.P., Shillito, B., Soyez, D., 2002. Structure and phylogeny of the crustacean hyperglycemic hormone and its precursor from a hydrothermal vent crustacean: The crab *Bythograea thermydron*. Peptides 23, 31–42. https://doi.org/10.1016/S0196-9781(01)00576-9

Tseng, D.-Y., Chen, Y.-N., Kou, G.-H., Lo, C.-F., Kuo, C.-M., 2001. Hepatopancreas is the extraovarian site of vitellogenin synthesis in black tiger shrimp, *Penaeus monodon*. Comparative Biochemistry and Physiology Part A 129, 909–917.

Tsutsui, N., Nagakura-Nakamura, A., Nagai, C., Ohira, T., Wilder, M.N., Nagasawa, H., 2013. The ex vivo effects of eyestalk peptides on ovarian vitellogenin gene expression in the kuruma prawn *Marsupenaeus japonicus*. Fisheries Science 79, 33–38. https://doi.org/10.1007/s12562-012-0566-5

Tuset, V.M., Espinosa, D.I., García-Mederos, A., Santana, J.I., González, J.A., 2011. Egg development and fecundity estimation in deep-sea red crab, *Chaceon affinis* (Geryonidae), off the Canary Islands (NE Atlantic). Fisheries Research 109, 373–378. https://doi.org/10.1016/j.fishres.2011.02.021

van Engel, W.A., 1958. The blue crab and its fishery in Chesapeake Bay: reproduction, early development, growth and mMigration. Commercial Fisheries Review 20.

van Heukelem, W., 1983. Growth of *Geryon quinquedens* (Brachyura: Geryonidae) juveniles in the laboratory. Fishery Bulletin 81, 903–905.

Ventura, T., Cummins, S.F., Fitzgibbon, Q., Battaglene, S., Elizur, A., 2014. Analysis of the central nervous system transcriptome of the Eastern rock lobster *Sagmariasus verreauxi* reveals its putative neuropeptidome. PLoS ONE 9. https://doi.org/10.1371/journal.pone.0097323

Ventura-López, C., Galindo-Torres, P.E., Arcos, F.G., Galindo-Sánchez, C., Racotta, I.S., Escobedo-Fregoso, C., Llera-Herrera, R., Ibarra, A.M., 2017. Transcriptomic information from Pacific white shrimp (*Litopenaeus vannamei*) ovary and eyestalk, and expression patterns for genes putatively involved in the reproductive process. General and Comparative Endocrinology 246, 164–182. https://doi.org/10.1016/J.YGCEN.2016.12.005

Vijayan, K.K., Reynolds, P., Mohanlal, D.L., Balasubramanium, C.P., 2013. Preliminary characterisation of gonad inhibiting hormone (GIH) gene and its expression pattern during vitellogenesis in giant tiger shrimp, *Penaeus monodon* Fabricius, 1798. Indian J Fish. 60, 103–107.

Wahle, R.A., Bergeron, C.E., Chute, A.S., Jacobson, L.D., Chen, Y., 2008. The Northwest Atlantic deep-sea red crab (*Chaceon quinquedens*) population before and after the onset of harvesting. ICES Journal of Marine Science 65, 862–872.

Wainwright, G., Webster, S.G., Wilkinson, M.C., Chung, J.S., Rees, H.H., 1996. Structure and Significance of mandibular organ-inhibiting hormone in the crab, *Cancer pagurus*: involvement in multihormonal regulation of growth and reproduction. Journal of Biological Chemistry 271, 12749–12754.

Wang, Z., Luan, S., Meng, X., Cao, B., Luo, K., Kong, J., 2019. Comparative transcriptomic characterization of the eyestalk in Pacific white shrimp (*Litopenaeus vannamei*) during ovarian maturation. General and Comparative Endocrinology 274, 60–72. https://doi.org/10.1016/j.ygcen.2019.01.002

Wang, Z., Sun, L., Guan, W., Zhou, C., Tang, B., Cheng, Y., Huang, J., Xuan, F., 2018. De novo transcriptome sequencing and analysis of male and female swimming crab (*Portunus trituberculatus*) reproductive systems during mating embrace (stage II). BMC Genetics 19. https://doi.org/10.1186/s12863-017-0592-5

Weinberg, J.R., Dahlgren, T.G., Trowbridge, N., Halanych, K.M., 2003. Genetic differences within and between species of deep-sea crabs (*Chaceon*) from the North Atlantic Ocean. Biological Bulletin 204, 318–326.

Weinberg, J.R., Keith, C., 2003. Population size-structure of harvested deep-sea red crabs (*Chaceon quinquedens*) in the northwest Atlantic Ocean. Crustaceana 79, 819–833.

Wigley, R.L., Theroux, R.B., Murray, H.E., 1975. Deep-sea red crab, *Geryon quinquedens*, survey off northeastern United States. Marine Fisheries Review 1154, 1–22.

Xu, Q., Liu, R., 2011. Development and characterization of microsatellite markers for genetic analysis of the swimming crab, *Portunus trituberculatus*. Biochemical Genetics 49, 202–212. https://doi.org/10.1007/s10528-010-9399-z

Yang, Y., Wang, J., Han, T., Liu, T., Wang, C., Xiao, J., Mu, C., Li, R., Yu, F., Shi, H., 2015. Ovarian transcriptome analysis of *Portunus trituberculatus* provides insights into genes

expressed during phase III and IV development. PLoS ONE 10. https://doi.org/10.1371/journal.pone.0138862

Yufang, S., Hongying, S., Yue, Z., Guanghui, Q., Qiang, Z., Naifa, L., 2009. Isolation and characterization of microsatellite loci in the freshwater crab *Sinopotamon yangtsekiense* and cross-species amplification in related taxa (Decapoda: Brachyura). Journal of Crustacean Biology 29, 413–418. https://doi.org/10.1651/08-3115.1

Zhang, Y., Jiang, S., Qiao, H., Xiong, Y., Fu, H., Zhang, W., Gong, Y., Jin, S., Wu, Y., 2021. Transcriptome analysis of five ovarian stages reveals gonad maturation in female *Macrobrachium nipponense*. BMC Genomics 22. https://doi.org/10.1186/s12864-021-07737-5

Zmora, N., Sagi, A., Zohar, Y., Chung, J.S., 2009a. Molt-inhibiting hormone stimulates vitellogenesis at advanced ovarian developmental stages in the female blue crab, *Callinectes sapidus* 2: novel specific binding sites in hepatopancreas and cAMP as a second messenger. Saline systems 5, 6. https://doi.org/10.1186/1746-1448-5-6

Zmora, N., Trant, J., Chan, S.M., Chung, J.S., 2007. Vitellogenin and its messenger RNA during ovarian development in the female blue crab, *Callinectes sapidus*: gene expression, synthesis, transport, and cleavage. Biology of Reproduction 77, 138–146. https://doi.org/10.1095/biolreprod.106.055483

Zmora, N., Trant, J., Zohar, Y., Chung, J.S., 2009b. Molt-inhibiting hormone stimulates vitellogenesis at advanced ovarian developmental stages in the female blue crab, *Callinectes sapidus* 1: an ovarian stage dependent involvement. Saline systems 5, 7. https://doi.org/10.1186/1746-1448-5-7

Chapter 2: Role of eyestalk neuropeptides in vitellogenesis of the red deep-sea crab, Chaceon quinquedens

Abstract

Eyestalk-derived neuropeptides, primarily the crustacean hyperglycemic hormone (CHH) neuropeptide family, regulate vitellogenesis in decapod crustaceans. This study aimed to understand the role of eyestalk-neuropeptides in vitellogenesis in this species with an extended intermolt stage. More specifically, the relationship between the molt-inhibiting hormone (MIH) and CHH levels, together with other eyestalk neuropeptides on the vitellogenic process in female *C. quinquedens*. Chromatography shows two CHH and one MIH peak in the sinus gland, with a CHH2 peak area four times larger than CHH1. The cDNA sequence of MIH and CHH of *C. quinquedens* isolated from the eyestalk ganglia and used in qPCR assay shows *MIH* is significantly higher only at ovarian stage 3 than 4 and 5, but *MIH* transcripts and neuropeptides do differ between stages 1 and 3. While *CHH* transcripts remain constant, their neuropeptide levels are higher at stages 3 than 1. Additionally, transcriptomic analysis of eyestalk ganglia at ovarian stages 1 and 3 found 28 eyestalk neuropeptides in the *de novo* assembly. Transcripts per million (TPM) values of ten neuropeptides increase by 1.3 to 2.0-fold at stage 3 compared to stage 1: two-fold for bursicon α, followed by CHH, RYamide, CCAP, allatostatin C, PDH1, and 1.3-fold of IDLSRF-like peptide, allatostatin A, PDH1. WXXXRamide, the only downregulated neuropeptide, its TPM decreases by ~2-fold at stage 3, compared to stage 1. Interestingly, neuroparsin with the highest TPM values remains the same in stages 1 and 3. The mandibular organ-inhibiting hormone is not found in *de novo* assembly. We report that CHH, MIH, and seven other neuropeptides may play a role in vitellogenesis in this species.

1. Introduction

The Atlantic red deep-sea crab, *Chaceon quinquedens* inhabits the Atlantic Ocean coastal waters from Nova Scotia to the Gulf of Mexico at depths ranging from 200 - 1800 meters (Chute

et al., 2008; Haefner and Musick, 1974; Steimle et al., 2001; Weinberg and Keith, 2003). It has supported a small commercial fishery along the eastern U.S. (from the Gulf of Maine to Cape Hatteras, NC) since the early 1970s (Wahle et al., 2008; Wigley et al., 1975). Currently, the *C. quinquedens* fishing industry harvests adult males based on size, with carapace widths greater than 94 mm (Chute et al., 2008), while females are protected from harvesting. The reproductive physiology of *C. quinquedens* with a life span of up to 15 years (Serchuk and Wigley, 1982), is largely unknown. Studies report that females exhibit a biennial reproductive cycle (Erdman et al., 1991; Hilário and Cunha, 2013; Martínez-Rivera and Stevens, 2020; Stevens and Guida, 2016), indicating a long-period vitellogenesis, and possibly a year-long embryogenesis like *Chionoecetes opilio* (Webb et al., 2007).

Vitellogenesis takes place in the hepatopancreas and ovary of decapod crustaceans and is known to be regulated largely by the neuropeptide hormone(s) derived from the eyestalk ganglia (Chen et al., 2018, 2014; Tsutsui et al., 2013; Zmora et al., 2009b, 2009a). Such neuropeptides, referred to as vitellogenesis-inhibiting/stimulating hormones (VI/SH) in shrimp and lobsters, belong to the crustacean hyperglycemic hormone (CHH) superfamily. In crab species, the multifunctional hormone CHH has a role in vitellogenesis, whereas lobsters and shrimp utilize a specific structural form of VI/SH (Chen et al., 2014; Kluebsoongnoen et al., 2020; Vijayan et al., 2013).

In decapod crustaceans, the X-organ and sinus gland located in the eyestalk ganglia synthesizes and release CHH family neuropeptides, respectively. But the neuropeptide profiles differ by species including structural variation in VI/SH or CHH. The SG of *C. quinquedens* is expected to have a neuropeptide profile similar to the green crab, *Carcinus maenas,* and

Callinectes sapidus (Chung and Webster, 1996; Zmora and Chung, 2014). Each of these crabs contains two structural isoforms of CHH and a single MIH.

Additionally, the molt-inhibiting hormone (MIH), another CHH superfamily member, also exhibits vitellogenesis-stimulating action in some species (Zmora et al., 2009b, 2009a). MIHs main role is to suppress the Y-organ (YO) inhibiting the synthesis and secretion of molting hormones in crustaceans. In the blue crab, *C. sapidus,* only females experience a puberty-terminal molt and remain at the intermolt stage for the remaining reproductively active stage of their lives, while males continue molting after reaching adulthood. In this case, MIH plays a dual role: halting the animal at intermolt by maintaining low hemolymph ecdysone levels and simultaneously stimulating vitellogenesis in the hepatopancreas (Zmora et al., 2009b, 2009a). Given this, it is plausible to suggest that the MIH in the female adult *C. quinquedens* that experience a 2 - 7 yearlong molt cycle (Gerrior, 1981; Lux et al., 1982) may play a similar dual role in regulating molting and stimulating vitellogenesis.

RNA sequencing (RNA-seq) has become a widely used tool for obtaining an overview of many physiological processes and gene discovery. Transcriptomic analysis has been used to identify putative neuropeptides in eyestalks of *Eriocheir sinensis* (Xu et al. 2015; Pang et al. 2019), *Homarus americanus* (Christie et al., 2017), *Litopenaeus vannamei* (Wei et al., 2020), *Macrobrachium rosenbergii* (Suwansa-Ard et al., 2015)**,** *Portunus trituberculatus* (Lv et al., 2017)*,* and *Procambarus clarkii* (Manfrin et al., 2015). Neuropeptides that may show reproductive functions are also reported in *Scylla paramamosain* (Bao et al., 2015; Gao et al., 2014), *E. sinensis* (X. Liu et al., 2019), *M. nipponense* (Qiao et al., 2017; Zhang et al., 2021), *P. trituberculatus* (Meng et al., 2015), *L. vannamei* (Wang et al., 2019), *Nephrops norvegicus*

(Rotllant et al., 2018), *Sagmariasus verreauxi* (Ventura et al., 2014), and *Cherax quadricarinatus* (Nguyen et al., 2016).

This study aimed to understand the role of eyestalk neuropeptide(s) in vitellogenesis. To this end, MIH and CHH cDNA sequences were first isolated from the eyestalk ganglia of *C. quinquedens* using traditional cloning methods and their expression was quantified in the eyestalk ganglia at various ovarian stages. *De novo* transcriptome assembly of the eyestalk ganglia was conducted to determine if transcripts of other neuropeptides differ by ovarian stages.

2. Materials and Methods

2.1. Animals

Adult female and male *C. quinquedens* at the intermolt stage were collected from the mid-Atlantic Bight region of the United States off the coast of Virginia up to Massachusetts. Samples were collected in the summer of 2013 onboard the NOAA R/V the Gordon Gunter and during the summers of 2014 and 2015 on the F/V Hannah Boden. We chose carapace length (CL) as the unit of measurement for the body size (Stevens and Guida, 2016), and the average size of the animals was 78 ± 2 mm (n = 5/ group) for expression analysis. Onboard the ship each female eyestalk was removed, and two small cuts were placed on each side of the eyestalk. The eyestalk was placed in a 1.5 mL microcentrifuge tube containing 500 µl of RNA*later*® Solution (Ambion®). Samples were then stored at -80°C until further processing. The remaining eyestalk was placed in a 1.5 mL microcentrifuge tube containing 1 mL Stefanini fixative (Stefanini et al., 1967).

Ovarian stages were determined according to the criteria described in (Haefner, 1977; Huang et al., 2020; Martínez-Rivera et al., 2020). In brief, ovary color and size were key parameters used to stage the ovary. Ovary stage by color is as follows, early *(*stage 1; colorless or

white to beige*)*, intermediate *(*stage 2; yellow to light orange*)*, advanced (stage 3; bright orange to brown), mature (stage 4; red-orange to purple), and redeveloping (stage 5; beige).

2.2. Separation of SG neuropeptides and dot blot assay

To determine the levels of CHH and MIH neuropeptides in the SG of adult male and female *C. quinquedens* (~90mm CL) were obtained from the Atlantic Red Crab Company (New Bedford, MA), including adult females (ovarian stage 2 and 3; n = 9) and males (n=11). A single SG of each animal was dissected and stored at -80°C until processed. The supernatant of a single SG that was briefly sonicated for 10 sec (Branson Sonifier 450) in 100 µl ice-cold 2N acetic acid and then centrifuged at 14,000 rpm for 3 min, the was separated on a Jupiter C18 column (4.6 x 150 mm, 5 µm, Phenomenex) connected to an RP-HPLC (Agilent 1100) at the gradient condition stated in Chung and Webster, (1996) and Zmora and Chung, (2014). Relative amounts of neuropeptides in a SG were calculated using chromatogram peak areas at 210 nm absorption, as reported for *C. borealis* (Chung et al., 2020). The major peaks were also collected manually and dried in a SpeedVac (Jouan) for a dot-blot assay. The peaks were tested using anti-*Callinectes* MIH and anti-*Carcinus* CHH sera to detect MIH and CHH cross-reactivity, respectively (Chung, 2004; Zmora et al., 2009a).

2.3. Localization of CHH family neuropeptides in the eyestalk

Whole-mount immunohistochemistry was used to localize CHH in the SG and X-organ neurosecretory cells of eyestalk ganglia (Chung and Webster, 2004). First, eyestalk ganglia stored in Stefanini fixative were carefully dissected under a dissecting microscope (Stefanini et al., 1967). Eyestalks were then washed in phosphate buffer containing Triton X (PTX) (50mM phosphate buffer + 0.5% Triton X + 0.01% sodium azide) 5 times for 30 minutes each and then incubated with *anti-Carcinus* CHH (Chung and Webster, 2004) serum at a final dilution of

1:1000 for 3 days at 4°C. Next, antibody-goat-anti rabbit-IgG labeled with Alexa 488 (Invitrogen 1:150) was applied to the samples after repeating the washing step. Finally, the images were collected using a Z-stack with a confocal microscope (Bio-Rad).

2.4. Isolation of CHH neuropeptide cDNA sequences

Total RNA from three eyestalks of adult females at ovarian stage 3 was extracted using QIAzol reagent (Qiagen) following the manufacturer's protocol. RNA quality and quantity were determined using a NanoDrop (Thermo Scientific, MA). One to 1.5 µg of total RNA was used to synthesize 5' and 3' RACE cDNAs using a SMART™ cDNA synthesis kit (TaKaRa). A two-step PCR was employed by following the procedures as described by (Chung et al., 2009; Vinagre et al., 2020; Vinagre and Chung, 2016; Zmora et al., 2009a) using the list of degenerate and gene-specific (Table 1) primers for *MIH*, *CHH*, and *vitellogenin* (*VTG*), with arginine kinase. Gene-specific primers were generated based on the initial sequencing and used for 5' and 3' RACE by following the procedures as described (Chung et al., 2012, 2009).

2.5. Sequence analysis and phylogenetic tree analysis

The full-length *CHH* and *MIH* nucleotide sequences were examined using NCBI BLASTX. Open reading frames (ORF) were identified via ORF finder (https://www.ncbi.nlm.nih.gov/orffinder/). The SignalP v.5.0 Server was used to determine the presence of a signal peptide (http://www.cbs.dtu.dk/services/SignalP/). RNA regulatory motifs were determined by examining the 5' and 3' UTR regions using RegRNA (http://regrna.mbc.nctu.edu.tw/index1.php; (Huang et al., 2006). Microsatellite Repeats Finder (http://insilico.ehu.es/mini_tools/microsatellites/?info) was used to identify microsatellite regions in the 3'UTR of *MIH*. The ORF sequences were also confirmed using a high fidelity Taq polymerase (Clonetch).

BLAST hits with greater than 75% sequence identity to other crab or decapod MIHs and CHHs (Appendix 1A and B) were selected and aligned using MAFFT, trimmed with GBLOCKS, and analyzed using PhyML with 100 bootstrap replicates as implemented by the phylogeny.fr website (https://www.phylogeny.fr).

2.6. Transcript levels of CHH, MIH, and VTG using qPCR assays

One to 1.5 µg of each eyestalk or hepatopancreas total RNA sample from adults with (78 ± 2 mm, CL) females (ovarian stages 2-5; n = 5/stage) and male eyestalk ganglia (101 ±1 mm, CL, n = 5) was reverse transcribed using a PrimeScript™ reagent kit with gDNA Eraser (TaKaRa) following the manufacturer's protocol. Each standard (*MIH, CHH, VTG,* and *AK*) was generated using published procedures (Chung et al., 2012, 2009). Transcript levels were estimated using cDNA samples (25 ng total RNA equivalent) in duplicate with Fast SYBR Green Master Mix (Life Technologies) on an Applied Biosystems 7500 Real-Time PCR System (Chung et al., 2012, 2009). The data were presented as mean ±1 SE transcripts/µg total RNA (n) where 'n' was the number of animals.

2.7. Sample preparation and Illumina Sequencing

Total RNA was extracted and quantified from the eyestalks of females at ovarian stages 1 and 3, as described above. Total RNA from three females at each stage (carapace length ≥73 mm) was pooled with an equal concentration. Pooled total RNA was used for library preparation and paired-end 150 base sequencing on the Illumina HiSeq 2500 platform (Macrogen).

2.8. De novo assembly and neuropeptide mining

The *de novo* assembly software Trinity (v. 2.8.6) was used to assemble paired-read sequences using the default parameters (including -trimmomatic to clean reads and no read normalization) (Grabherr et al., 2011; Haas et al., 2013). The completeness of the assembly was

evaluated using the Benchmarking Universal Single-Copy Orthologs (BUSCO) approach by aligning to the arthropod reference set (Simão et al., 2015). Expression levels of the transcripts in the assemblies were estimated by remapping the high-quality reads on both transcriptomes separately with RNA-Seq by Expectation-Maximization (Robinson et al., 2010, Li and Dewey, 2011). RSEM data for both stages were further analyzed to determine the most highly expressed sequences. Additionally, differential expression using edgeR at p-value (0.05) and log fold change >2 was used.

The eyestalk transcriptome was mined for several neuropeptides using a well-established workflow (Christie, 2016, 2015, 2014a; Christie et al., 2017, 2008; Christie and Pascual, 2016; Ma et al., 2010). The amino acid sequences of 53 eyestalk-neuropeptides from different decapod species were collected and placed in a text file using NCBIs efetch utility (Supplemental data 2). These sequences were then BLASTed against the eyestalk assembly using an e-value cut-off 1e-30. The TPM values for the neuropeptides reported were extracted from the RSEM data to examine differences between ovarian stages.

Candidate neuropeptide nucleotide sequences were then compared to GenBank using BLASTX (https://blast.ncbi.nlm.nih.gov/). As described above, contigs detected as neuropeptides were translated using ExPASy Translate (web.expasy.org/translate) and signal peptides were identified with SignalP v.5.0 Server (http://www.cbs.dtu.dk/services/SignalP/).

2.9. Statistical analysis

The statistical significance of each dataset was examined using a one-way ANOVA with the post-hoc Kruskal-Wallis test or a Student's *t-test*. Significant differences were accepted at $P < 0.05$ and noted with different letters and 'asterisks. The data are presented as mean ± SE (n), where 'n' is the number of animals.

3. Results

3.1. Identification of CHH and MIH and their levels in the SG and the localization of CHH in the eyestalk ganglia

The three peaks that were manually collected from an RP-HPLC and labeled as 1, 2, and 3 were cross-reacted with anti-*Carcinus* CHH and -*Callinectes* MIH sera and referred to as CHH1 (no. 1), CHH2 (no. 2) and MIH (no.3) (Fig. 2.1A; noted with a dot under the peak).

The function of CHH and MIH in vitellogenesis was examined by comparing their levels in the SG of adult females (n = 10) and males (n = 11). As shown in Fig. 2.1B, the neuropeptide peak area (m*AU) was ranked: the highest was CHH2 with 5143 ± 523 in females, significantly higher ($P < 0.05$) than 3594 ± 489 in males. The CHH precursor-related peptide (CPRP) had a peak area of 2222 ± 22 in females and 1768 ± 223 in males, while CHH1 was 918 ± 74 in females and 893 ± 133 in males. MIH was 895 ± 57 in females and 795 ± 102 in males. The ratio of CHH1:CHH2 was 1:4 in both females and males, rendering CHH2 as the major isoform.

3.2. MIH & CHH cDNA sequence analyses

The full-length *MIH* cDNA sequence (1561 bp, GenBank accession no. MK558261), includes a 454 bp 5’ UTR, a 342 bp ORF, and a 765 bp 3’ UTR (Fig. 2.2A). The ORF encodes a 113 amino acid (aa) prohormone, including a 35-aa signal peptide (italicized) followed by a 78-aa mature hormone (underlined) (Fig. 2.2A). The deduced peptide sequence of MIH is depicted in a schematic diagram (Fig. 2.2B). The red crab MIH aa sequence is 86.7% identical to *C. maenas* (Q27225.1).

Phylogenetic analysis of MIH sequences placed *C. quinquedens* between two clades corresponding to Cancroidea and Portunoidae (Fig. 2.3). The Portunidae MIHs formed a well-supported clade (0.97 bootstrap) The *C. quinquedens* MIH was sister to a weakly supported Cancroidae (0.72 bootstrap), which was grouped with weak bootstrap support (0.75) with

Portunidae. Finally, these two clades were placed with strong support (0.99) with the last brachyuran clade, including *E. sinensis*, *Gecarcinus lateralis*, and *Discoplax celeste*. The outgroup consists of the clade containing lobster, shrimp, and crayfish.

The full-length cDNA sequence of *C. quinquedens* CHH (*CHH*: 957 bp, GenBank accession no. QBC16325.1) contains a 5' UTR (360 bp), an ORF (420 bp,) and a 3' UTR (177 bp). The ORF finder predicts that the preprohormone encodes a 140-aa sequence containing a 20-aa signal peptide (italicized), 42-aa CPRP (dotted line), a dibasic cleavage site (KR) (boxed), a 72-aa mature hormone (double underline), an amidation site (G) (circled), and a tribasic cleavage site (RKK) (boxed) followed by a stop codon (Fig. 2.4A). A schematic diagram shows the major components including the signal peptide, CPRP, followed by the mature CHH peptide (Fig. 2.4B). The 5' UTR contains several translation regulatory sites: two Terminal Oligopyrimidine Tracts (TOP), one upstream open reading frame (uORF), and one Gamma interferon-activated inhibitor of Ceruloplasmin mRNA translation (GAIT element). No regulatory motifs were found in the 3' UTR of the *CHH* sequence (Table 1). Its amino acid sequence is 90.14% identical to *C. maenas* CHH (CAA35605.1).

Phylogenetic analysis of *C. quinquedens* CHH is more complex with multiple structural isoforms found in *Cancer productus* with four isoforms, *Pachygraspus marmoratus* with two, and within the genus *Homarus* two (Fig. 2.5). The *C. quinquedens* CHH was placed outside a strongly supported clade of mostly portunids (0.97) with *C. maenas*. The Portunidae was sister to a moderately supported Cancridae clade (0.86) and formed a monophyletic group with other brachyurans (0.98 support). In a weakly supported position to Portunoidae species (0.70 bootstrap), the superfamilies, Cancroidae and Majoidea were moderately supported (0.84 bootstrap) (Fig. 2.5).

3.3. Transcript levels of MIH, CHH, and VTG using qPCR assays

The *MIH* transcripts of adult female eyestalks at ovarian stages 2 - 5 (n = 5/stage) were estimated using qPCR and compared with males (n = 7) (Fig. 2.6B). *MIH* transcripts increased from 4.3 ± 1.1 E6 transcripts/µg total RNA at stage 2 to 7.6 ± 1.2 E6 transcripts/µg total RNA at stage 3, although these did not differ statistically. However, stage 3 levels were significantly higher than stage 4 (2.3 ± 0.3 E6 transcripts/µg total RNA) and stage 5 (3.3 ± 0.6 E6 transcripts/µg total RNA). Adult male *MIH* expression averaged 4.0 ± 1.2 E6· transcripts/µg total RNA and was high in comparison to females at stages 3 - 5.

CHH expression in the eyestalk ganglia of adult females at ovarian stages 2 - 5 (n = 5/stage) was also estimated (Fig. 2. 6A). The CHH expression did not significantly differ among ovarian stages, varying from stage 2 with 4.6 ± 0.9 E6 transcripts/µg total RNA; 2.1 ± 0.6 E6 transcripts/µg total RNA at stage 3; 2.5 ± 0.8 E6 transcripts/µg total RNA at stage 4; and 3.3 ± 0.1 E6 transcripts/µg total RNA at stage 5. AK levels, as a reference gene did not differ by stages, ranging from 2.5 ± 0.6 E5 transcripts/µg total RNA, the lowest at stage 2 to 5.5 ± 0.6 E5 transcripts/µg total RNA, the highest at stage 5.

The *VTG* transcripts in the hepatopancreas obtained from the same animals at ovarian stages 2 - 5 were measured (Fig. 2.6C). There was an increasing trend in *VTG* levels among ovarian stages 2, 3, and 4, although they did not differ significantly. At stage 2, VTG was 1.7 ± 1.4 E7 transcripts/µg total RNA at stage 3 with 5.6 ± 0.2 E7 transcripts/µg total RNA and stage 4 with 7.8 ± 0.2 E7 transcripts/µg total RNA. At stage 5, *VTG* decreased to 1.7 ± 0.4 E7 transcripts/µg total RNA, similar to ovarian stage 2. AK levels, as a reference gene remained consistent through all stages, ranging from 1.1 ± 0.4 E7 transcripts/µg total RNA to 1.6 ± 0.3 E7 transcripts/µg total RNA.

3.4. Transcriptome: Quality of RNAseq, de novo assembly, and differential expression analysis

The *de novo* assembly of the 37 million paired-end reads at stage 1 and 38 million pairs at stage 3 of eyestalk ganglia produced 159,079 contigs (isoforms) with 98,355 genes (Table 2). The assembly was aligned to the 1066 genes in the arthropod BUSCO reference and was 77.1% complete and 26.0% of the contigs were duplicated (Table 2) (C:77.1%[S:51.1%,D:26.0%],F:13.4%,M:9.5%,n:1066). Alignment of reads back to the assembled transcriptome showed 84.8% of stage 1 and 84.4% of stage 3 reads were concordantly mapped. The number of differentially expressed genes with TPM values greater than one was 8,756 unique to stage 1 and 9,528 to stage 3 while, 56,613 were shared (Supplemental data 3).

The *de novo* assembly contained 3 MIH isoforms, two of which contained full-length coding sequences (Supplemental data 4) and 7 CHH isoforms which differed only in their 3' UTR length and sequence (Supplemental data 5). The MIH sequences found in the *de novo* assembly and the cloned MIH were identical in the ORF. Similarly, the cloned and assembled CHH ORF were identical.

3.5. Neuropeptide prediction

Twenty-eight neuropeptides out of 53 search candidates were identified from the *C. quinquedens* eyestalk Trinity assembly transcriptome based on high identity sequences and full-length matches with e-values less than E-30 (Supplemental data 6). These 28 varied in expression levels with the highest TPM values of 1981.7 for neuroparsin to the lowest, 0.2 for bursicon α subunit (Table 4). The TPM values of these 28 were compared at stages 1 and 3, the majority (26/28), including MIH, ranged from 0.75 to 1.3-fold change, considered to be constant (Table 4). Ten neuropeptides had TPM values higher at stage 3 than 1, ranging from the highest with a two-fold change of bursicon α, CHH, adipokinetic hormone (AKH)/corazonin-like,

RYamide, crustacean cardioactive peptide (CCAP), allatostatin C, to the least with 1.37, IDLSRF-like peptide, allatostatin A, and PDH1. WXXXRamide was downregulated by ~2 fold at stage 3, compared to stage1 (Table 4).

4. Discussion

The red deep-sea crab, *C. quinquedens* of the Geryonidae family, inhabits the deep water between 200 – 1800 m along the continental shelf and slopes of the Atlantic Ocean (Haefner and Musick, 1974; Serchuk, 1977; Wahle et al., 2008; Wigley et al., 1975), with temperatures ranging from 5 to 8˚C and little to no sunlight. Large adults spend up to 7 years at the intermolt stage, with a long molt cycle (Gerrior, 1981; Lux et al., 1982), suggesting that this species is a k-selection strategist (Taylor and Roterman, 2017). A biannual reproductive cycle was proposed (Martínez-Rivera et al., 2020; Stevens and Guida, 2016) and with an intermolt phase lasting up so long, adult females could potentially undergo at least 3 separate vitellogenesis events within a single molt cycle. Interestingly, no adult females at the previtellogenic stage are found in this study as stage 1 is noted as early vitellogenic (Haefner, 1978). In *C. quinquedens*, it is assumed that vitellogenesis is a continuous process throughout adult life. Unlike *C. quinquedens,* adult female *C. sapidus* experience pubertal-terminal molt and remains in the intermolt stage while actively engaging in vitellogenesis and other reproductive activities facilitated by high levels of MIH (Zmora et al., 2009b, 2009a).

In decapod crustaceans, the molt cycle is largely regulated by two neuropeptide hormones produced in eyestalk ganglia, MIH and CHH (Webster et al., 2012). Given the established roles of CHH and MIH, these two neuropeptides were described in depth using multiple techniques in *C. quinquedens*. First, their levels were measured in the eyestalk with HPLC and immunostaining of the SG. Then the cDNA for these two neuropeptide hormones was

cloned, sequenced, and transcript levels measured with qPCR across four different ovarian stages. Finally, transcriptomics of female *C. quinquedens* eyestalk ganglia, the neuropeptide hub, at two ovarian stages was also used to provide a broad view of neuropeptides in *C. quinquedens*. The overall goal is to compare these results with the established roles of CHH and MIH in other valuable crustacean species.

There are hundreds of CHH family neuropeptides deposited in GenBank, with significantly higher numbers of CHH than MIH, most of which are reported from warm-water crustacean species. The phylogenetic position with *C. productus*, *C. maenas*, and *Metacarcinus magister*, *Chaceon* MIH is closely related to an intertidal species, *C. maenas*, with an 87% sequence identity. *C. quinquedens* CHH sequence is grouped with other cold-water crabs (Fig. 5), such as snow and cancer crab species. Like MIH, *C. quinquedens* CHH is most closely related to *C. maenas* with a 90% sequence identity.

Like some crab species, *C. sapidus, C. maenas*, and *C. bairdi*, *Chaceon* SG has two forms of CHH (1 and 2) at a 1:4 ratio, with CHH2 as the major form (Chung et al., 2009; Jia et al., 2012). Interestingly, this ratio of 1:4 of CHH1:CHH2 is higher than other species, including *C. sapidus* 1:5; *C. maenas* 1:8; *C. pagurus* 1:10 (Chung et al., 1998; Chung and Webster, 2003), possibly due to a slower conversion of Q to <Q at the position 1 of CHH primary sequence. In addition, in contrast to *C. maenas* where the SG of males and females has similar CHH content (Chung and Webster, 1996), in *C. quinquedens*, CHH2 content was consistently higher in females than males of the same size.

Hormones involved in energy metabolism tend to accumulate in the glands of crustaceans and insects. In decapod crustaceans, the CHH tends to accumulate in the SG throughout the life cycle of *C. sapidus* (Chung, unpublished data) since the first detection in early embryogenesis

(Chung and Webster, 2004; Techa et al., 2015). Insect adipokinetic hormone shows a similar profile of accumulation in *corpora cardiaca* of *Locusta migratoria* (O'Shea and Rayne, 1992).

The analysis of the 5' and 3'UTR suggests that *Chaceon* CHH contains several potential 5' UTR regulatory sites, but none in the 3'UTR. This finding agrees with earlier reports on other cold-water crab species, *Chionoecetes* spp. (Chung et al., 2015, 2009), and further supports the translational regulation of CHH in cold-water crustacean species (Chung et al., 2015). Unlike CHH, for MIH, there are no translation regulatory sites in the 5' and 3' UTRs in *C. quinquedens*; possibly, the transcription and translation would be related and proportional.

The SG CHH content differed between ovarian stages, without significant changes in CHH transcript levels based on qPCR. In transcriptome analysis, CHH has higher TPM values at stage 3 than 1 among neuropeptides with TPM values >1. Thus, when considering translation regulatory sites in the 5'UTR of CHH cDNA sequence, transcription levels may not intimately reflect translation. The difference in CHH neuropeptide levels in the SG at different ovarian stages likely indicates release into the hemolymph.

On the other hand, significant changes in MIH transcripts, but not neuropeptide levels at different ovarian stages, were found. MIH and CHH levels of hemolymph are not measured in this study, while their levels would be high as all the females were at the intermolt stage, as reported. The correlation between MIH and VTG expression levels could imply that it may play a role in vitellogenesis. In *C. quinquedens*, both MIH and CHH may play a role in vitellogenesis. The hepatopancreas is the primary tissue for vitellogenesis and contributes >99% of VTG for ovarian development in crabs, including *C. quinquedens* and *C. sapidus* (Huang et al., 2020; Zmora et al., 2007).

The *C. quinquedens* eyestalk transcriptome provided a more comprehensive picture of the various hormones and their expression in this critical tissue at different vitellogenic stages. A particular interest is investigating genes expressed in stage 1 females which are harder to access due to fishery regulation and depth limits. The *C. quinquedens* transcriptome is valid, showing high BUSCO scores (77.1%) compared to the arthropod genes. To date, several reproductive hormones in the eyestalk have been detected in lobsters, shrimp, crabs, crayfish, and crayfish using a transcriptomic approach (Lv et al., 2017; Nguyen et al., 2016; Pang et al., 2019; Wei et al., 2020). Similarly, our study reports on multiple reproductive neuropeptides derived from the eyestalk.

Similar to studies that previously mined for potential neuropeptides (Christie, 2016, 2014a, 2014b, 2014c; Christie et al., 2008), our search yielded 53 eyestalk-borne neuropeptides. The inventory of *C. quinquedens* neuropeptides is similar to those reported in the mud crab, *S. paramamosain* (Bao et al., 2015), with a particular finding of the neuropeptides WXXXRamide and GSEFamide. Even hormones with similar TPM values at different ovarian stages could still have a role in vitellogenesis. This study focuses on the changes in TPMs by ovarian stages. CHH, the only neuropeptide, has higher TPM values at stage 3 than 1 among neuropeptides with TPM values >100. Neuroparsin ranks the top with the highest TPM values and remains at the same levels at ovarian stages 1 and 3. The constant expression of neuroparsin in *C. quinquedens* suggests a role in vitellogenesis and warrants further study to determine if it plays a negative role as seen in other decapods (Badisco et al., 2011; Liu et al., 2020; Yang et al., 2014).

Crustacean Female Sex Hormone (CFSH) is listed as the fourth most highly expressed reproductive neuropeptide in the *Chaceon* transcriptome. In *C. quinquedens*, CFSH is slightly higher in the eyestalks of females at ovarian stage 1. The function of CFSH is first reported in *C.*

sapidus for developing adult-specific morphological features required for the mating and brooding of embryos (Zmora and Chung, 2014). *Chaceon* belonging to pleocyemata broods' embryos for their development up to 9-10 months until hatching (Elner et al., 1987; Haefner, 1978); it is reasonable to suggest that CFSH has the same functions as found in other crab species (Jiang et al., 2020; Zmora and Chung, 2014).

In *C. quinquedens*, MOIH is not found in the SG or *de novo* assembly, which is not unusual as MOIH is found only in Cancridae crabs (Chung et al., 2020; Wainwright et al., 1996). Interestingly, *de novo* assembly contains several forms of insect allatostatins, the function of which is equivalent to crustacean MOIH, inhibiting methyl farnesoate (MF) synthesis by the mandibular organ (A. Liu et al., 2019; Wainwright et al., 1996). Therefore, the role of MF in the vitellogenesis of the hepatopancreas of decapod crustaceans needs to be further studied, because it may play a role in ovarian vitellogenesis and maturation (Medesani et al., 2012; Reddy and Reddy, 2015). Therefore, further studies into the lack of MOIH in decapod crustacean species and the potential of allatostatins to inhibit MF synthesis is warranted.

5. Conclusion

The current study reports the isolation of *MIH* and *CHH* cDNA sequences, their expression levels, and the presence of MIH and CHH neuropeptide in the eyestalk ganglia-SG complex. The assembled transcriptome of female eyestalk ganglia during ovarian development also other neuropeptides. Taken together, both MIH and CHH are traditionally known to display molt-inhibiting activity and may play an important role in the vitellogenesis of females in species such as *C. quinquedens* with long intermolt periods (2 - 7 years). In future studies, hemolymph titers of MIH and CHH need to be determined throughout ovarian development to define further their role in vitellogenesis and reproduction in this species.

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References

Badisco, L., Marchal, E., van Wielendaele, P., Verlinden, H., Vleugels, R., vanden Broeck, J., 2011. RNA interference of insulin-related peptide and neuroparsins affects vitellogenesis in the desert locust *Schistocerca gregaria*. Peptides 32. https://doi.org/10.1016/j.peptides.2010.11.008

Bao, C., Yang, Y., Huang, H., Ye, H., 2015. Neuropeptides in the cerebral ganglia of the mud crab, *Scylla paramamosain*: transcriptomic analysis and expression profiles during vitellogenesis. Scientific Reports 5. https://doi.org/10.1038/srep17055

Chen, T., Ren, C., Jiang, X., Zhang, L., Li, H., Huang, W., Hu, C., 2018. Mechanisms for type-II vitellogenesis-inhibiting hormone suppression of vitellogenin transcription in shrimp hepatopancreas: Crosstalk of GC/cGMP pathway with different MAPK-dependent cascades. PLOS ONE 13. https://doi.org/10.1371/journal.pone.0194459

Chen, T., Zhang, L.-P., Wong, N.-K., Zhong, M., Ren, C.-H., Hu, C.-Q., 2014. Pacific white shrimp (<i>Litopenaeus vannamei<I/>) vitellogenesis-inhibiting hormone (VIH) Is predominantly expressed in the brain and negatively regulates hepatopancreatic vitellogenin (VTG) gene expression. Biology of Reproduction 90, 1–10. https://doi.org/10.1095/biolreprod.113.115030

Christie, A.E., 2016. Expansion of the neuropeptidome of the globally invasive marine crab *Carcinus maenas*. General and Comparative Endocrinology 235, 150–169. https://doi.org/10.1016/j.ygcen.2016.05.013

Christie, A.E., 2015. Neuropeptide discovery in *Symphylella vulgaris* (Myriapoda, Symphyla): In silico prediction of the first myriapod peptidome. General and Comparative Endocrinology 223, 73–86. https://doi.org/10.1016/j.ygcen.2015.09.021

Christie, A.E., 2014a. Prediction of the peptidomes of *Tigriopus californicus* and *Lepeophtheirus salmonis* (Copepoda, Crustacea). General and Comparative Endocrinology 201, 87–106. https://doi.org/10.1016/j.ygcen.2014.02.015

Christie, A.E., 2014b. Expansion of the *Litopenaeus vannamei* and *Penaeus monodon* peptidomes using transcriptome shotgun assembly sequence data. General and Comparative Endocrinology 206, 235–254. https://doi.org/10.1016/j.ygcen.2014.04.015

Christie, A.E., 2014c. Identification of the first neuropeptides from the Amphipoda (Arthropoda, Crustacea). General and Comparative Endocrinology 206, 96–110. https://doi.org/10.1016/j.ygcen.2014.07.010

Christie, A.E., Cashman, C.R., Stevens, J.S., Smith, C.M., Beale, K.M., Stemmler, E.A., Greenwood, S.J., Towle, D.W., Dickinson, P.S., 2008. Identification and cardiotropic actions of brain/gut-derived tachykinin-related peptides (TRPs) from the American lobster *Homarus americanus*. Peptides 29, 1909–1918. https://doi.org/10.1016/j.peptides.2008.07.010

Christie, A.E., Pascual, M.G., 2016. Peptidergic signaling in the crab Cancer borealis: Tapping the power of transcriptomics for neuropeptidome expansion. General and Comparative Endocrinology 237, 53–67. https://doi.org/10.1016/j.ygcen.2016.08.002

Christie, A.E., Roncalli, V., Cieslak, M.C., Pascual, M.G., Yu, A., Lameyer, T.J., Stanhope, M.E., Dickinson, P.S., 2017. Prediction of a neuropeptidome for the eyestalk ganglia of the lobster *Homarus americanus* using a tissue-specific de novo assembled transcriptome. General and Comparative Endocrinology 243, 96–119. https://doi.org/10.1016/j.ygcen.2016.11.001

Chung, J., Christie, A., Flynn, E., 2020. Molecular cloning of crustacean hyperglycemic hormone (CHH) family members (CHH, molt-inhibiting hormone, and mandibular organ-inhibiting hormone) and their expression levels in the Jonah crab, *Cancer borealis*. General and Comparative Endocrinology 295, 113522. https://doi.org/10.1016/j.ygcen.2020.113522

Chung, J.S., Ahn, I.S., Yu, O.H., Kim, D.S., 2015. Crustacean hyperglycemic hormones of two cold water crab species, *Chionoecetes opilio,* and *C. japonicus*: isolation of cDNA sequences and localization of CHH neuropeptide in eyestalk ganglia. General and Comparative Endocrinology 214, 177–185. https://doi.org/10.1016/j.ygcen.2014.08.017

Chung, J.S., Bembe, S., Tamone, S., Andrews, E., Thomas, H., 2009. Molecular cloning of the crustacean hyperglycemic hormone (CHH) precursor from the x-organ and the identification of the neuropeptide from sinus gland of the Alaskan Tanner crab, *Chionoecetes bairdi*. General and Comparative Endocrinology 162, 129–133. https://doi.org/10.1016/j.ygcen.2009.03.012

Chung, J.S., Katayama, H., Dircksen, H., 2012. New functions of arthropod bursicon: inducing deposition and thickening of new cuticle and hemocyte granulation in the blue crab, *Callinectes sapidus*. PLoS ONE 7. https://doi.org/10.1371/journal.pone.0046299

Chung, J.S., Webster, S.G., 2004. Expression and release patterns of neuropeptides during embryonic development and hatching of the green shore crab, *Carcinus maenas*. Development (Cambridge, England) 131, 4751–61. https://doi.org/10.1242/dev.01312

Chung, J.S., Webster, S.G., 2003. Moult cycle-related changes in biological activity of moult-inhibiting hormone (MIH) and crustacean hyperglycaemic hormone (CHH) in the crab, *Carcinus maenas*: from target to transcript. European Journal of Biochemistry 270, 3280–3288. https://doi.org/10.1046/j.1432-1033.2003.03720.x

Chung, J.S., Webster, S.G., 1996. Does the N-Terminal Pyroglutamate Residue have Any Physiological Significance for Crab Hyperglycemic Neuropeptides? European Journal of Biochemistry 240, 358–364. https://doi.org/10.1111/j.1432-1033.1996.0358h.x

Chung, J.S., Wilkinson C, M., Webster, S.G., 1998. Amino acid sequences of both isoforms of crustacean hyperglycemic hormone (CHH) and corresponding precursor-related peptide in *Cancer pagurus*. Regulatory Peptides 77, 17–24. https://doi.org/10.1016/S0167-0115(98)00024-X

Chute, A., Jacobson, L., Rago, P., MacCall, A., 2008. Deep Sea red crab. Northeast Data Poor Stocks Working Group Meeting 1–18.

Elner, R.W., Koshio, S., Hurley, G.V., 1987. Mating behavior of the deep-sea red crab, *Geryon quinquedens* Smith (Decapoda, Brachyura, Geryonidae). Crustaceana 52, 194–201.

Erdman, R.B., Blake, N.J., Lockhart, F.D., Lindberg, W.J., Perry, H.M., Waller, R.S., 1991. Comparative reproduction of the deep-sea crabs *Chaceon fenneri* and *C. quinquedens* (Brachyura: Geryonidae) from the northeast Gulf of Mexico. Invertebrate reproduction & development 19, 175–184.

Gao, J., Wang, X., Zou, Z., Jia, X., Wang, Y., Zhang, Z., 2014. Transcriptome analysis of the differences in gene expression between testis and ovary in green mud crab (*Scylla paramamosain*). BMC Genomics 15. https://doi.org/10.1186/1471-2164-15-585

Gerrior, P., 1981. The distribution and effects of fishing on the deep-sea red crab, *Geryon quinquedens* Smith, off southern New England. North Dartmouth, MA.

Grabherr, M.G., Haas, B.J., Yassour, M., Levin, J.Z., Thompson, D.A., Amit, I., Adiconis, X., Fan, L., Raychowdhury, R., Zeng, Q., Chen, Z., Mauceli, E., Hacohen, N., Gnirke, A., Rhind, N., di Palma, F., Birren, B.W., Nusbaum, C., Lindblad-Toh, K., Friedman, N., Regev, A.,

2011. Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nature Biotechnology 29, 644–652. https://doi.org/10.1038/nbt.1883

Haas, B.J., Papanicolaou, A., Yassour, M., Grabherr, M., Blood, P.D., Bowden, J., Couger, M.B., Eccles, D., Li, B., Lieber, M., Macmanes, M.D., Ott, M., Orvis, J., Pochet, N., Strozzi, F., Weeks, N., Westerman, R., William, T., Dewey, C.N., Henschel, R., Leduc, R.D., Friedman, N., Regev, A., 2013. De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. Nature Protocols 8, 1494–1512. https://doi.org/10.1038/nprot.2013.084

Haefner, P., 1978. Seasonal aspects of the biology, distribution and relative abundance of the deep-sea red crab *Geryon quinquedens* Smith, in the vicinity of the Norfolk Canyon, Western North Atlantic. Proceedings of the National Shellfisheries Association 68, 49–62.

Haefner, P.A., 1977. Reproductive biology of the female Deep-Sea Red Crab, *Geryon quinquedens*, from the Chesapeake Bight. Fishery Bulletin 75, 91–102.

Haefner, P.A., Musick, J.A., 1974. Observations on Distribution and Abundance of Red Crabs in Norfolk Canyon and Adjacent Continental Slope. Marine Fisheries Review 36, 31–34.

Hilário, A., Cunha, M.R., 2013. Notes on a mating event of the deep-sea crab Chaceon affinis in the Gorringe Bank (NE Atlantic). Deep-Sea Research Part II: Topical Studies in Oceanography 92, 58–62. https://doi.org/10.1016/j.dsr2.2013.01.021

Huang, H.Y., Chien, C.H., Jen, K.H., Huang, H. da, 2006. RegRNA: An integrated web server for identifying regulatory RNA motifs and elements. Nucleic Acids Research 34, 429–434. https://doi.org/10.1093/nar/gkl333

Huang, X., Green, S., Sook Chung, J., 2020. The presence of an insulin-like peptide-binding protein (ILPBP) in the ovary and its involvement in the ovarian development of the red deep-sea crab, *Chaceon quinquedens*. General and Comparative Endocrinology 301, 113653. https://doi.org/10.1016/j.ygcen.2020.113653

Jia, C., Hui, L., Cao, W., Lietz, C.B., Jiang, X., Chen, R., Catherman, A.D., Thomas, P.M., Ge, Y., Kelleher, N.L., Li, N., 2012. High-definition de novo sequencing of Crustacean Hyperglycemic Hormone (CHH)-family neuropeptides. Molecular and Cellular Proteomics 11, 1951–1964. https://doi.org/10.1074/mcp.M112.020537

Jiang, Q., Lu, B., Lin, D., Huang, H., Chen, X., Ye, H., 2020. Role of crustacean female sex hormone (CFSH) in sex differentiation in early juvenile mud crabs, *Scylla paramamosain*. General and Comparative Endocrinology 289. https://doi.org/10.1016/j.ygcen.2019.113383

Kluebsoongnoen, J., Panyim, S., Udomkit, A., 2020. Regulation of vitellogenin gene expression under the negative modulator, gonad-inhibiting hormone in *Penaeus monodon*. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 243, 110682.

Liu, A., Liu, F., Shi, W., Huang, H., Wang, G., Ye, H., 2019. C-Type allatostatin and its putative receptor from the mud crab serve an inhibitory role in ovarian development. Journal of Experimental Biology 222. https://doi.org/10.1242/jeb.207985

Liu, J., Liu, A., Liu, F., Huang, H., Ye, H., 2020. Role of neuroparsin 1 in vitellogenesis in the mud crab, *Scylla paramamosain*. General and Comparative Endocrinology 285, 113248. https://doi.org/10.1016/j.ygcen.2019.113248

Liu, X., Ma, K.Y., Liu, Z.Q., Feng, J. bin, Ye, B.Q., Qiu, G.F., 2019. Transcriptome analysis of the brain of the Chinese mitten crab, *Eriocheir sinensis*, for neuropeptide abundance profiles during ovarian development. Animal Reproduction Science 201, 63–70. https://doi.org/10.1016/j.anireprosci.2018.12.010

Lux, F.E., Ganz, A.R., Rathjen, W.F., 1982. Marking studies on the red crab *Geryon quinquedens* Smith off southern New England. Journal of Shellfish Research 2, 71–80.

Lv, J., Zhang, L., Liu, P., Li, J., 2017. Transcriptomic variation of eyestalk reveals the genes and biological processes associated with molting in *Portunus trituberculatus*. PLoS ONE 12. https://doi.org/10.1371/journal.pone.0175315

Ma, M., Gard, A.L., Xiang, F., Wang, J., Davoodian, N., Lenz, P.H., Malecha, S.R., Christie, A.E., Li, L., 2010. Combining in silico transcriptome mining and biological mass spectrometry for neuropeptide discovery in the Pacific white shrimp Litopenaeus vannamei. Peptides 31, 27–43. https://doi.org/10.1016/j.peptides.2009.10.007

Manfrin, C., Tom, M., de Moro, G., Gerdol, M., Giulianini, P.G., Pallavicini, A., 2015. The eyestalk transcriptome of red swamp crayfish *Procambarus clarkii*. Gene 557, 28–34. https://doi.org/10.1016/j.gene.2014.12.001

Martínez-Rivera, S., Long, W.C., Stevens, B.G., 2020. Physiological and behavioral sexual maturity of female red deep-sea crabs *Chaceon quinquedens* (Smith, 1879) (Decapoda:

Brachyura: Geryonidae) in the Mid-Atlantic Bight. Journal of Crustacean Biology 40, 330–340. https://doi.org/10.1093/jcbiol/ruaa007

Martínez-Rivera, S., Stevens, B.G., 2020. Embryonic development and fecundity of the red deep-sea crab Chaceon quinquedens (Smith, 1879) (Decapoda: Brachyura: Geryonidae) in the Mid-Atlantic Bight determined by image analysis. Journal of Crustacean Biology 40, 230–236. https://doi.org/10.1093/jcbiol/ruaa017

Medesani, D.A., Ferré, L.E., Grodzielski, M., Rodríguez, E.M., 2012. *In vitro* effect of methyl farnesoate on the vitellogenin content of ovary and hepatopancreas, in the crayfish *Cherax quadricarinatus*. Invertebrate Reproduction & Development 56. https://doi.org/10.1080/07924259.2011.582691

Meng, X.L., Liu, P., Jia, F.L., Li, J., Gao, B.Q., 2015. De novo transcriptome analysis of *Portunus trituberculatus* ovary and testis by RNA-Seq: Identification of genes involved in gonadal development. PLoS ONE 10. https://doi.org/10.1371/journal.pone.0128659

Nguyen, T.V., Cummins, S.F., Elizur, A., Ventura, T., 2016. Transcriptomic characterization and curation of candidate neuropeptides regulating reproduction in the eyestalk ganglia of the Australian crayfish, *Cherax quadricarinatus*. Scientific Reports 6, 1–19. https://doi.org/10.1038/srep38658

O'Shea, M., Rayne, R.C., 1992. Adipokinetic hormones: cell and molecular biology. Experientia 48, 430–438.

Pang, Y. yang, Zhang, C., Xu, M. jie, Huang, G. yong, Cheng, Y. xu, Yang, X. zhen, 2019. The transcriptome sequencing and functional analysis of eyestalk ganglions in Chinese mitten crab (*Eriocheir sinensis*) treated with different photoperiods. PLoS ONE 14, 1–18. https://doi.org/10.1371/journal.pone.0210414

Qiao, H., Fu, H., Xiong, Y., Jiang, S., Zhang, W., Sun, S., Jin, S., Gong, Y., Wang, Y., Shan, D., Li, F., Wu, Y., 2017. Molecular insights into reproduction regulation of female Oriental River prawns *Macrobrachium nipponense* through comparative transcriptomic analysis. Scientific Reports 7, 1–11. https://doi.org/10.1038/s41598-017-10439-2

Reddy, B.S., Reddy, P.R., 2015. Methyl farnesoate induced ovarian vitellogenin syntehsis in freshwater rice field crab *Oziothelphusa senex senex* Fabricious. Journal of Infertility and Reproductive Biology 3, 237–244.

Rotllant, G., Nguyen, T.V., Hurwood, D., Sbragaglia, V., Ventura, T., Company, J.B., Joly, S., Elizur, A., Mather, P.B., 2018. Evaluation of genes involved in Norway lobster (*Nephrops norvegicus*) female sexual maturation using transcriptomic analysis. Hydrobiologia 825, 137–158. https://doi.org/10.1007/s10750-018-3521-3

Serchuk, F.M., 1977. Assessment of red crab (*Geryon quinquedens*) populations in the northwest Atlantic. Northeast Fisheries Science Center Ref. Doc. 77, 1–15.

Serchuk, F.M., Wigley, R.L., 1982. Deep-sea red crab, *Geryon quinquedens*. Fish Distribution 15, 125–129.

Simão, F.A., Waterhouse, R.M., Ioannidis, P., Kriventseva, E. v., Zdobnov, E.M., 2015. BUSCO: Assessing genome assembly and annotation completeness with single-copy orthologs. Bioinformatics 31, 3210–3212. https://doi.org/10.1093/bioinformatics/btv351

Stefanini, M., de Martino, C.E.S.A.R.E., Zamboni, L., 1967. Fixation of ejaculated spermatozoa for electron microscopy. Nature 216, 173–174.

Steimle, F.W., Zetlin, C.A., Chang, S., 2001. Red deepsea crab, *Chaceon (Geryon) quinquedens*, life history and habitat characteristics., NOAA Tech. Memo. NMFS-NE-163.

Stevens, B.G., Guida, V., 2016. Depth and temperature distribution, morphometrics, and sex ratios of red deepsea crab (*Chaceon quinquedens*) at 4 sampling sites in the Mid-Atlantic Bight. Fishery Bulletin 114, 343–359. https://doi.org/10.7755/FB.114.3.7

Suwansa-Ard, S., Thongbuakaew, T., Wang, T., Zhao, M., Elizur, A., Hanna, P.J., Sretarugsa, P., Cummins, S.F., Sobhon, P., 2015. In silico neuropeptidome of female *Macrobrachium rosenbergii* based on transcriptome and peptide mining of eyestalk, central nervous system and ovary. PLoS ONE 10, 1–31. https://doi.org/10.1371/journal.pone.0123848

Taylor, M.L., Roterman, C.N., 2017. Invertebrate population genetics across Earth's largest habitat: the deep-sea floor. Molecular Ecology 26, 4872–4896. https://doi.org/10.1111/mec.14237

Techa, S., Alvarez, J. v., Sook Chung, J., 2015. Changes in ecdysteroid levels and expression patterns of ecdysteroid-responsive factors and neuropeptide hormones during the embryogenesis of the blue crab, *Callinectes sapidus*. General and Comparative Endocrinology 214, 157–166. https://doi.org/10.1016/j.ygcen.2014.07.017

Tsutsui, N., Nagakura-Nakamura, A., Nagai, C., Ohira, T., Wilder, M.N., Nagasawa, H., 2013. The ex vivo effects of eyestalk peptides on ovarian vitellogenin gene expression in the

kuruma prawn *Marsupenaeus japonicus*. Fisheries Science 79, 33–38. https://doi.org/10.1007/s12562-012-0566-5

Ventura, T., Cummins, S.F., Fitzgibbon, Q., Battaglene, S., Elizur, A., 2014. Analysis of the central nervous system transcriptome of the Eastern rock lobster *Sagmariasus verreauxi* reveals its putative neuropeptidome. PLoS ONE 9. https://doi.org/10.1371/journal.pone.0097323

Vijayan, K.K., Reynolds, P., Mohanlal, D.L., Balasubramanium, C.P., 2013. Preliminary characterisation of gonad inhibiting hormone (GIH) gene and its expression pattern during vitellogenesis in giant tiger shrimp, *Penaeus monodon* Fabricius, 1798. Indian J Fish. 60, 103–107.

Vinagre, A.S., Chung, J.S., 2016. Effects of starvation on energy metabolism and crustacean hyperglycemic hormone (CHH) of the Atlantic ghost crab *Ocypode quadrata* (Fabricius, 1787). Marine Biology 163. https://doi.org/10.1007/s00227-015-2797-3

Vinagre, A.S., Model, J.F.A., Vogt, É.L., Manara, L.M., Trapp, M., da Silva, R.S.M., Chung, J.S., 2020. Diet composition and long-term starvation do not affect crustacean hyperglycemic hormone (CHH) transcription in the burrowing crab *Neohelice granulata* (Dana, 1851). Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology 247. https://doi.org/10.1016/j.cbpa.2020.110738

Wahle, Richard A, Bergeron, Charlene E, Chute, A.S., Jacobson, L.D., Chen Wahle, Y., Wahle, R A, Bergeron, C E, 2008. The Northwest Atlantic deep-sea red crab (*Chaceon quinquedens*) population before and after the onset of harvesting. ICES Journal of Marine Science 65, 862–872.

Wainwright, G., Webster, S.G., Wilkinson, M.C., Chung, J.S., Rees, H.H., 1996. Structure and Significance of mandibular organ-inhibiting hormone in the crab, *Cancer pagurus*: involvement in multihormonal regulation of growth and reproduction. Journal of Biological Chemistry 271, 12749–12754.

Wang, Z., Luan, S., Meng, X., Cao, B., Luo, K., Kong, J., 2019. Comparative transcriptomic characterization of the eyestalk in Pacific white shrimp (*Litopenaeus vannamei*) during ovarian maturation. General and Comparative Endocrinology 274, 60–72. https://doi.org/10.1016/j.ygcen.2019.01.002

Webb, J.B., Eckert, G.L., Shirley, T.C., Tamone, S.L., 2007. Changes in embryonic development and hatching in *Chionoecetes opilio* (Snow Crab) with variation in incubation temperature. Biol. Bull. 213, 67–75.

Webster, S.G., Keller, R., Dircksen, H., 2012. The CHH-superfamily of multifunctional peptide hormones controlling crustacean metabolism, osmoregulation, moulting, and reproduction. General and Comparative Endocrinology 175, 217–233. https://doi.org/10.1016/j.ygcen.2011.11.035

Wei, C., Pan, L., Zhang, X., Tong, R., 2020. Comparative transcriptome analysis of eyestalk from the white shrimp *Litopenaeus vannamei* after the injection of dopamine. Gene 763, 145115. https://doi.org/10.1016/j.gene.2020.145115

Weinberg, J.R., Keith, C., 2003. Population size-structure of harvested deep-sea red crabs (*Chaceon quinquedens*) in the northwest Atlantic Ocean. Crustaceana 79, 819–833.

Wigley, R.L., Theroux, R.B., Murray, H.E., 1975. Deep-sea red crab, Geryon quinquedens, survey off northeastern United States. Marine Fisheries Review 1154, 1–22.

Yang, S.P., He, J.-G., Sun, C.B., Chan, S.F., 2014. Characterization of the shrimp neuroparsin (MeNPLP): RNAi silencing resulted in inhibition of vitellogenesis. FEBS Open Bio 4. https://doi.org/10.1016/j.fob.2014.09.005

Zhang, Y., Jiang, S., Qiao, H., Xiong, Y., Fu, H., Zhang, W., Gong, Y., Jin, S., Wu, Y., 2021. Transcriptome analysis of five ovarian stages reveals gonad maturation in female *Macrobrachium nipponense*. BMC Genomics 22. https://doi.org/10.1186/s12864-021-07737-5

Zmora, N., Chung, J.S., 2014. A novel hormone is required for the development of reproductive phenotypes in adult female crabs. Endocrinology 155, 230–239. https://doi.org/10.1210/en.2013-1603

Zmora, N., Sagi, A., Zohar, Y., Chung, J.S., 2009a. Molt-inhibiting hormone stimulates vitellogenesis at advanced ovarian developmental stages in the female blue crab, *Callinectes sapidus* 2: novel specific binding sites in hepatopancreas and cAMP as a second messenger. Saline systems 5, 6. https://doi.org/10.1186/1746-1448-5-6

Zmora, N., Trant, J., Chan, S.M., Chung, J.S., 2007. Vitellogenin and its messenger RNA during ovarian development in the female blue crab, *Callinectes sapidus*: gene expression, synthesis,

transport, and cleavage. Biology of Reproduction 77, 138–146. https://doi.org/10.1095/biolreprod.106.055483

Zmora, N., Trant, J., Zohar, Y., Chung, J.S., 2009b. Molt-inhibiting hormone stimulates vitellogenesis at advanced ovarian developmental stages in the female blue crab, *Callinectes sapidus* 1: an ovarian stage dependent involvement. Saline systems 5, 7. https://doi.org/10.1186/1746-1448-5-7

Tables:

Table 1. Primer used for clone and expression of CHH, MIH, VTG, and AK. List of primers for isolating the cDNA sequence of *CHH, MIH*, *VTG,* and *AK* from the eyestalk of female *C. quinquedens* and qPCR assays.

Primer Name	Primer sequences (5' to 3')
CHHdF1	TGYAARGGNGTNTAYGA
CHHdF2	CATRCAYTGNCKRAANAC
CHHdR1	GAYTTYATHGCNGCNGGNAT
MIHdF1	GNGTNATHAAYGAYGA
MIHdF2	TGYCCNAAYYTNATHGGNAA
MIHdR1	AARTCYTCRTTRAARAARCA
AKdF1	CAYTTYYTNTTYAARGARGG
AKdF2	AAYGCNTGYMGNTAYTGGCC
AKdR1	CCNCCYTCNGCYTCNGTRTG
ChqAK-QF	CTGGGCCAGGTATACCGCCGCCTTGTCAGC
ChqAK-QR	GGGGAGCTTGATGTGGACGGAGGCACGCAC
ChqMIH-QF2	GAACAGAGACCTTTATAAGAAAGTAGAGTG
ChqMIH-QR2	CTGACCCACCGCTTCAACTGTGTCATCTCT
ChqChh-QF1	CGATCAGCGCAAGGATTAAGAATGGAGCGG
ChqChh-QR2	CCAGGTCGCTGAACAGGCCACGGTCATACA
TaqvitF	TGTACAGCTGAAAGGCGTGG
TaqvitR	CATGGGCCGAGAACAGTCA
ChqVTG-QF	ATCTCCCGCATTGGTCAGGACCAGCTGGTG
ChqVTG-QR	GGCATCGGCTTCAACTCCATCCCTGGTGAT

Abbreviations: d=degenerate primers; Q= primers used for qPCR assays

Table 2. Eyestalk transcriptome statistics.

Total reads Eyestalk Stage 1	74,613,704 (37,306,852 paired)
Total reads Eyestalk Stage 3	75,395,282 (37,697,641 paired)
Total	150,008,986
Percent GC	43.65
Isoform Statistics	
Number of transcripts	159,079
Average transcript size (bp)	652
N50	931
Total assembled bases	103,875,774
BUSCO	(C:77.1%[S:51.1%,D:26.0%],F:13.4%,M:9.5%,n:1066)
Gene Statistics	
Total number of genes	98,335
Average gene size (bp)	598
N50	803
Total assembled bases	58,839,147

Table 3. **Regulatory sites in select crustaceans.** Comparison of putative translational regulatory sites in the 5' and 3' UTR of cold and warm water decapods (RNAreq analysis).

	Regulatory sites in 5'UTR				**Regulatory sites in 3'UTR**		
Species	**TOP**	**IRES**	**uORF**	**GAIT**	**GAIT**	**SECIS-T1**	**CPE**
C. quinquedens	**2**		**1**	**1**			
C. bairdi	**2**						
C. japonicus	**5**		**1**	**1**	**1**		
C. opilio	**5**		**1**	**1**	**1**		
C. sapidus							
S. paramamosain	**1**				**3**	**1**	**1**
S. olivacea		**1**		**1**	**8**	**1**	**1**

Abbreviations: TOP= Terminal Oligopyrimidine Tracts, IRES=, uORF= upstream Open Reading Frame, GAIT= Gamma Interferon Activated Inhibitor of Ceruloplasmin mRNA translation, SECIS-T1= Selenocysteine Insertion Sequence Type-1, CPE= Cytoplasmic Polyadenylation Element.

Table 4. Eyestalk neuropeptides identified from eyestalk transcriptome of *C. quinquedens*.

Neuropeptide name	TPM (stage)		FPKM (stages)		TPM fold change	FPKM fold change	bit score	Trinity ID
	1	3	1	3				
Neuroparsin	1981.7	2138.0	3024.5	3332.2	1.08	1.10	115	DN72010_c0_g1_i1
Corazonin	649.7	536.9	991.6	836.8	0.83	0.84	162	DN1204_c0_g1_i2
EH	499.6	470.7	762.5	733.6	0.94	0.96	195	DN45_c0_g1_i5
PDH1	**329.8**	**452.0**	**503.2**	**704.5**	**1.37**	**1.40**	576	DN21903_c0_g2_i1
CCAP	**260.1**	**397.7**	**397.0**	**619.8**	**1.53**	**1.56**	245	DN16410_c0_g1_i1
MIH	223.7	168.5	341.3	262.5	0.75	0.77	207	DN3681_c0_g1_i1
DH31	146.3	165.9	223.3	258.6	1.13	1.16	186	DN7150_c0_g1_i2
CHH	**107.4**	**193.7**	**163.9**	**301.8**	**1.80**	**1.84**	177	DN365_c0_g1_i6
Orcokinin	97.8	103.2	149.3	160.8	1.06	1.08	186	DN2600_c0_g3_i1
SIFamide	89.8	81.2	137.1	126.6	0.90	0.92	92.8	DN701_c7_g1_i1
Myosuppressin	75.7	76.4	115.5	119.0	1.01	1.03	133	DN1178_c0_g1_i1
IDLSRF-LP	**47.8**	**65.5**	**73.0**	**102.2**	**1.37**	**1.40**	**307**	DN4730_c0_g1_i3
Tachykinin	39.4	46.1	60.1	71.8	1.17	1.19	142	DN8282_c0_g1_i1
CFSH	38.0	31.8	58.0	49.52	0.83	0.85	446	DN51890_c0_g1
Short NP-F	**26.2**	**34.6**	**40.0**	**53.9**	**1.32**	**1.35**	92.8	DN4036_c0_g1_i1
Allostatin B	20.5	21.6	31.3	33.7	1.05	1.08	479	DN2210_c0_g1_i7
FLRFamide	14.5	17.8	22.2	27.7	1.23	1.25	562	DN8457_c0_g1_i2
Allatostatin C	13.2	11.5	20.1	18.0	0.88	0.90	180	DN7628_c0_g1_i3
Allatostatin A	**10.6**	**14.0**	**16.2**	**21.7**	**1.32**	**1.34**	222	DN1313_c1_g1_i4
Glycoprotein B	**11.0**	**15.4**	**16.7**	**23.9**	**1.40**	**1.43**	234	DN7316_c0_g1_i5
GSEFLamide	9.7	9.3	14.8	14.4	0.96	0.98	345	DN11505_c0_g1_i15
RYamide	8.0	6.2	12.2	9.7	0.78	0.79	164	DN7666_c0_g2_i1
AKH/ corazonin-like	**4.3**	**7.6**	**9.2**	**12.6**	**1.75**	**1.36**	148	DN3516_c1_g1_i1
Pyrokinin	**5.6**	**8.9**	**7.5**	**14.1**	**1.59**	**1.89**	120	DN3453_c0_g4_i1
Glycoprotein A2	3.7	2.8	5.7	4.3	0.74	0.75	214	DN6268_c2_g1_i1
proNP F I	3.6	4.5	5.5	7.1	1.26	1.29	137	DN4769_c0_g1_i2
WXXXRamide	***3.5***	***1.9***	***5.4***	***3.0***	***0.55***	***0.56***	538	DN11635_c0_g1_i1
Bursicon Alpha	**0.2**	**0.4**	**0.3**	**0.6**	**2.00**	**2.00**	100	DN60850_c0_g1_i1

13 neuropeptides are higher at stage 3 than 1 (bolded values).
Abbreviations: EH= eclosion hormone; DH= diuretic hormone; CCAP= crustacean cardioactive peptide; AKH= adipokinetic hormone

Figures:

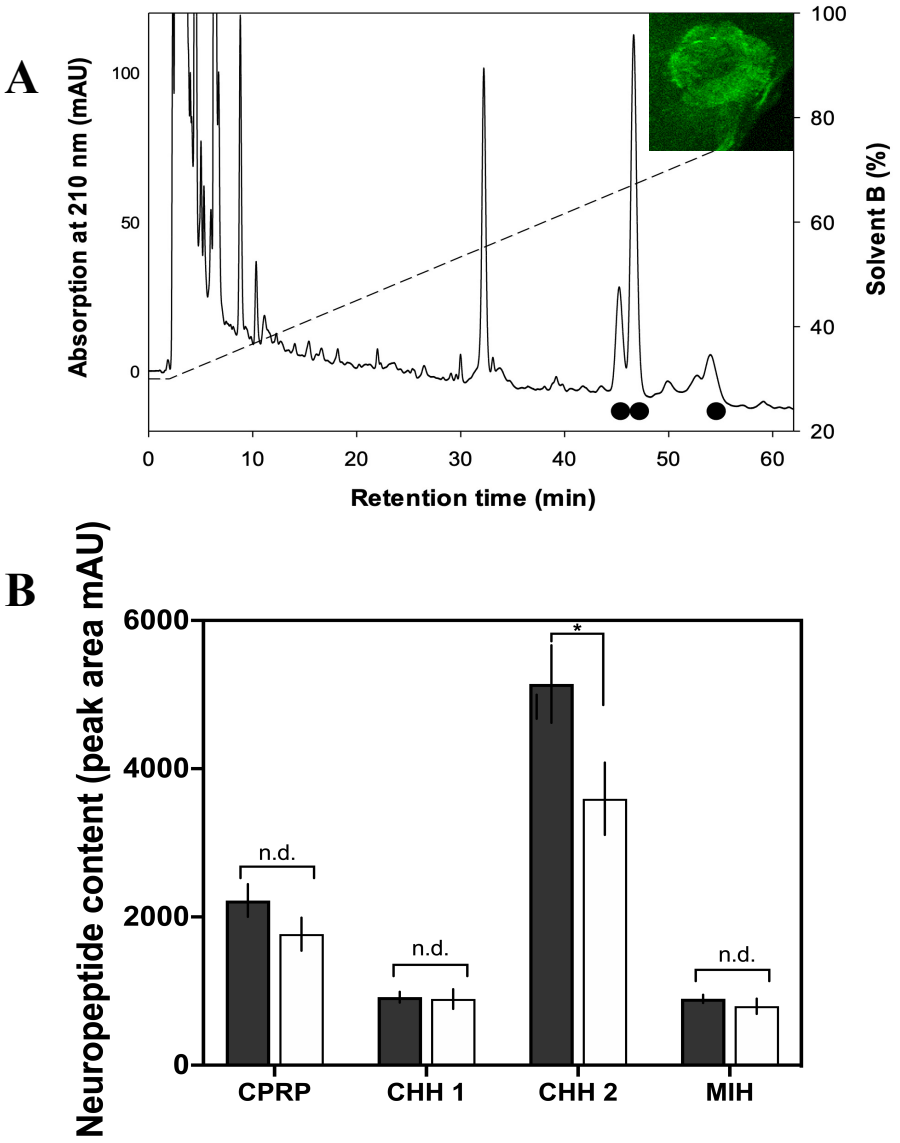
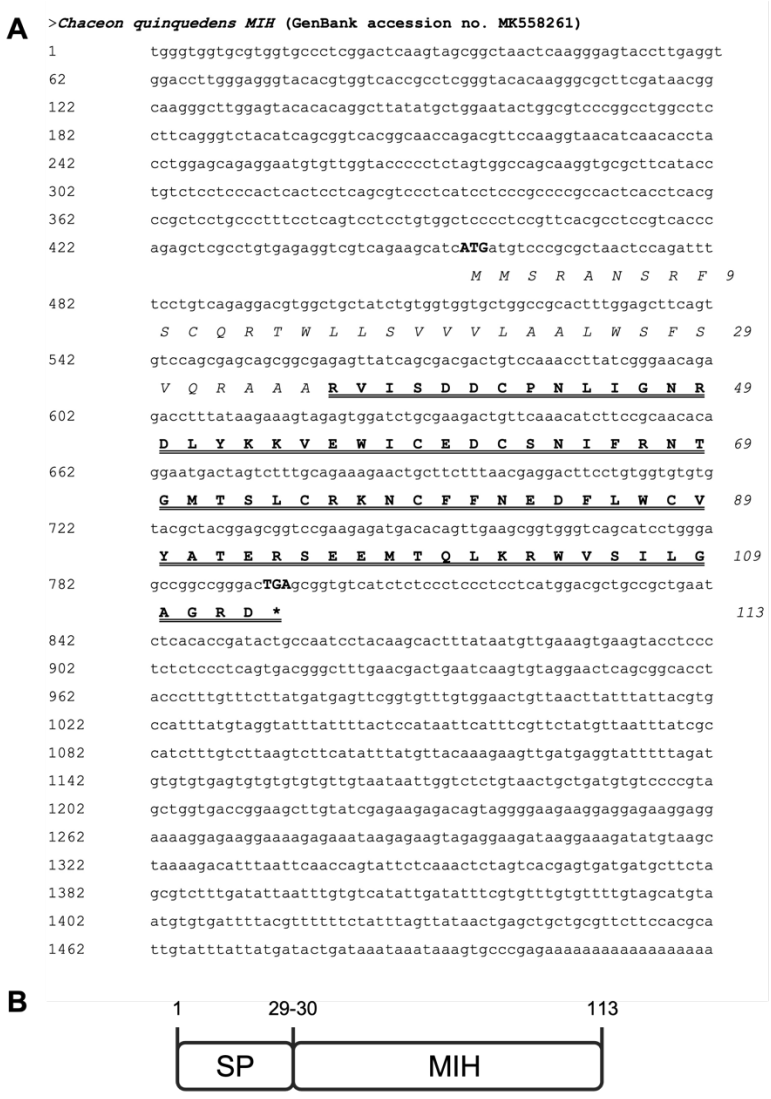


Fig. 2.1. Relative quantification of neuropeptide levels in the sinus gland of adult female and male red deep-sea crabs. (**A**). A single sinus gland was separated on a C18 Gemini column with a gradient of 40–80% B (0.1% TFA in 60% acetonitirle + 40% water) over 60 min at 0.6 ml/min flow rate. The detection was at 210 nm wavelength that monitors peptide bonds. Neuropeptide peaks were identified as CHH 1, CHH 2, and MIH after identifying them with dot blot assays with specific antisera (noted with black dots). The inset shows a SG with CHH immunostaining. (**B**) Neuropeptide content (peak area) for CPRP, CHH1, CHH2, and MIH. Dark bars are females and open bars are males. The data are presented as mean ± 1SE (n). A student's

t-test was used to determine statistical significance at $P < 0.05$. Differences were indicated with



"*".

Fig. 2.2. Nucleotide and amino acid sequence of ChqMIH. The full-length MIH cDNA sequence of *C. quinquedens* (*ChqMIH*, MK558261). The numbers on the left-hand side correspond to nucleotides and those on the right-hand side to the putative amino acid sequence of CHH identified using ORF finder (http://www.ncbi.nlm.nih.gov/projects/gorf/). The start codon (ATG) is shown in bold and capitalized. The 35 aa signal peptide was predicted by Signal P (http://www.cbs.dtu.dk/services/SignalP/) and is italicized. The putative MIH is noted in bold and has a bold underline, and the stop codon is bolded and capitalized (TGA) and marked with ''*'' underneath. **(B)** A schematic diagram of the putative MIH with numbers for the position of aa: the signal peptide: 1 - 35; and mature MIH: 36 – 113.

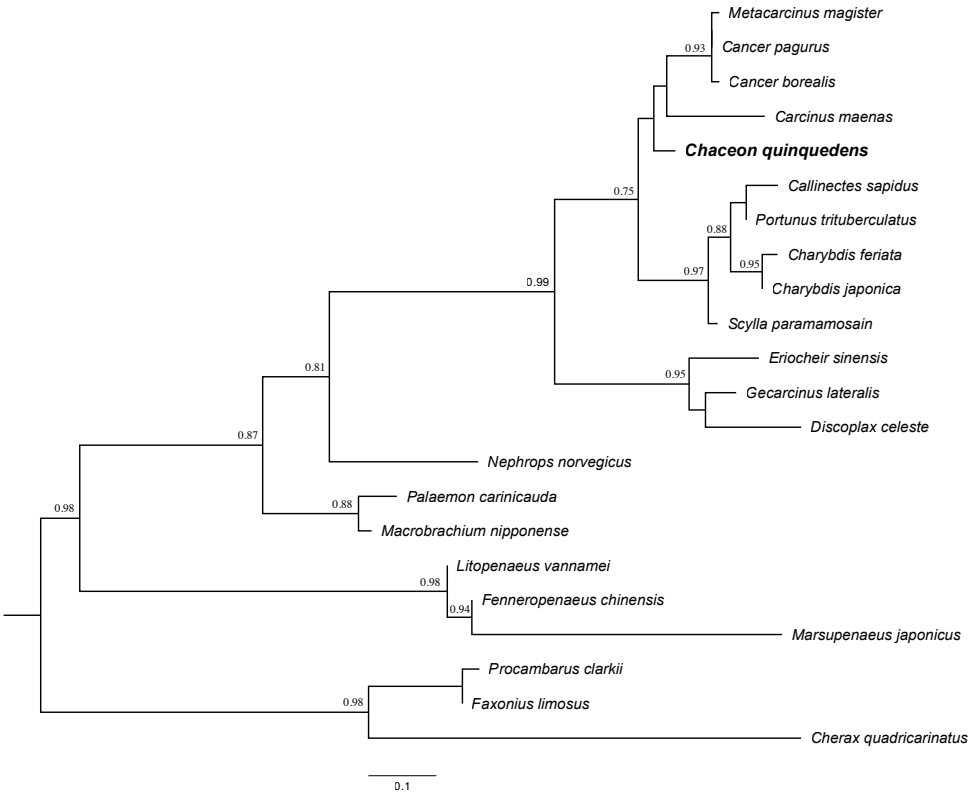


Fig. 2.3. Phylogenetic tree of MIH amino acid sequence of *C. quinquedens* and other decapods. A multiple sequence alignment of MIH amino acid sequences from other decapods and *C. quinquedens* (Supplemental data 1A) was used to generate a phylogenetic tree using Phylogeny fr. ChqMIH sequence is bolded. In brief, sequences were aligned using MUSCLE, trimmed with GBLOCKS, and the tree was built with PhyML.

A

>*Chaceon quinquedens CHH* (GenBank accession no. MG786421)

```
1     gtatatatattataaaatttacacacaaaaaagatatatgcatgagtatgccccgatata
61    taggtgacactatagagaatacacgatatatatataccgcgcggggagatctctcatata
121   ttggagacgcacgcgggcgcgagtacactagagagtgagcacagggatatatacagagag
181   tgcgggcgcgcacacacacacacacacacacacacacacacacacacacacacacacaca
241   cacacactaacacacacacgcacatatcctcttcttgaataccttaatgtttgaccatct
301   ctctctctctctctctctctctctctctctctctctctctctctctctcagaacagcttccccc
361   ATGatggcagtactggtaatggtgtgcgtattcatgtacacactaccaaacgcacacgcc
       M  M  A  V  L  V  M  V  C  V  F  M  Y  T  L  P  N  A  H  A  20
421   cgatcagcgcaaggattaagaatggagcggcttttggcttcactgcgaggcccatcggac
       R  S  A  Q  G  L  R  M  E  R  L  L  A  S  L  R  G  P  S  D  40
481   accgcatctccccttggggatttcagcggctctagtaatcagggctcatcctctcacccc
       T  A  S  P  L  G  D  F  S  G  S  S  N  Q  G  S  S  S  H  P  60
541   ttggagaagcgacagatttacgacacgtcctgcaagggagtgtatgaccgtggcctgttc
       L  E  K  R  Q  I  Y  D  T  S  C  K  G  V  Y  D  R  G  L  F  80
601   agcgacctggagcatgtgtgtgacgactgctacaacctctaccgtacctcctacgtggcc
       S  D  L  E  H  V  C  D  D  C  Y  N  L  Y  R  T  S  Y  V  A  100
661   agtgcttgcagatcggactgctacagtaacttggtgttccggcaatgtatgaacgacctc
       S  A  C  R  S  D  C  Y  S  N  L  V  F  R  Q  C  M  N  D  L  120
721   ctcctcatggaagactttgaccagtacgccggaagagtacagatggttggaaggaagaag
       L  L  M  E  D  F  D  Q  Y  A  G  R  V  Q  M  V  G  R  K  K  140
781   TAGaatgtctatataatattgctacagtactagagctctccccctatcgcctctcacaac
       *
841   acgggaaagagagttataaatgcctatataatattactattccctccccatatcgcctct
901   cacaagtgccaaatattagagaaagagcaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa
```

B

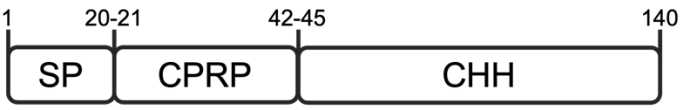


Fig. 2.4. Nucleotide and amino acid sequence of ChqCHH.
(**A**) The full-length CHH cDNA sequence of *C. quinquedens* (*ChqCHH*, MG786421). The numbers on the left-hand side correspond to nucleotides and those on the right-hand side to the putative amino acid sequence of CHH identified using ORF finder (http://www.ncbi.nlm.nih.gov/projects/gorf/). The start codon (ATG) is bolded and capitalized. The 20 aa signal peptide was predicted by Signal P (http://www.cbs.dtu.dk/services/SignalP/) is italicized. The putative CPRP (42 aa) has a dotted underline. A dibasic cleavage site (KR) is boxed. The putative CHH is noted in bold and has a double underline, together with amidation site (G) which is also circled, a boxed tribasic cleavage site (RKK) and the stop codon is bolded (TAG) marked with ‘‘*’’. (**B**) A schematic diagram of the putative prepro CHH numbers for the position of aa: the signal peptide: 1 - 20; CPRP: 21 - 42; a dibasic cleavage site: 43 - 44; and CHH: 45 - 140.

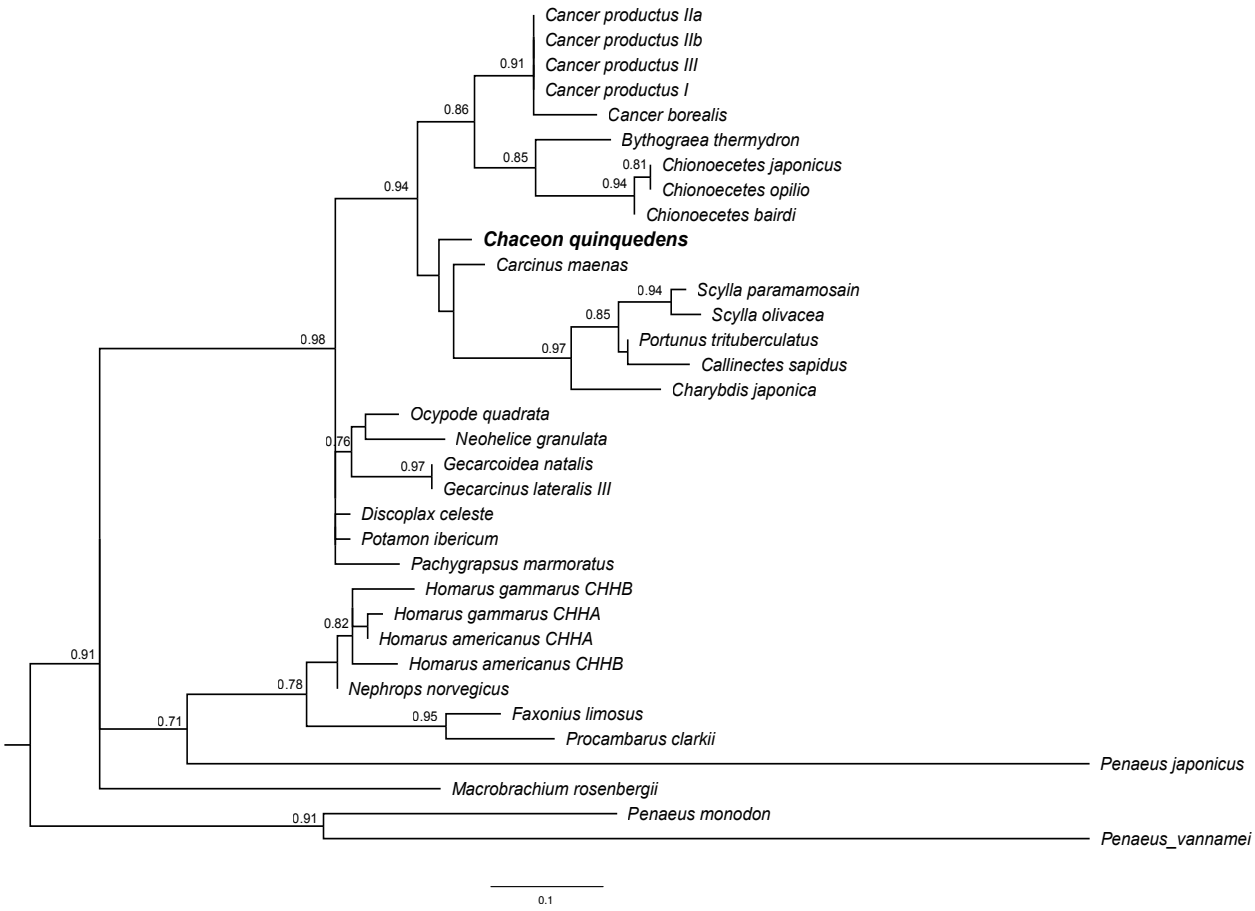


Fig. 2.5. Phylogenetic tree of eyestalk CHH amino acid sequence of *C. quinquedens* and of other decapods. A multiple sequence alignment of eyestalk CHH amino acid sequences from other decapods and *C. quinquedens* (Supplemental data 1B) was used to generate a phylogenetic tree using Phylogeny fr. ChqCHH sequence is bolded. In brief, sequences were aligned using MUSCLE, trimmed with GBLOCKS, and the tree was built with PhyML.

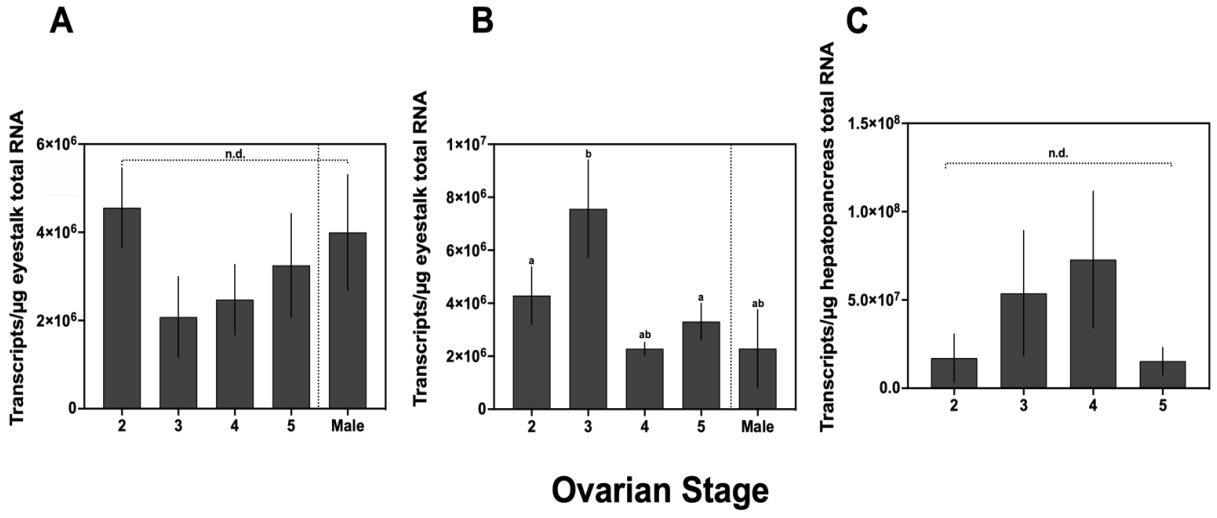


Fig. 2.6. Neuropeptide and vitellogenin expression levels from *C. quinquedens* adult

females. Expression levels of eyestalk CHH (**A**) and MIH (**B**) in adult females (ovarian stages 2 - 5) and adult males. (**C**) Expression levels of vitellogenin synthesized in female (ovarian stages 2 - 5) hepatopancreas. All data are shown as mean ± 1 SE transcripts/µg total RNA. Each cDNA sample was assayed in duplicate. Statistical significance was established using a non-parametric one-way analysis of variance (ANOVA) (Kruskal-Wallis test). Statistical differences at $P < 0.05$ were indicated with letters.*Supplemental data*

Supplemental 1 – Amino acid sequences used for Phylogenetic tree generation

Supplemental 2 - Crustacean eyestalk neuropeptide sequence search

Supplemental 3 - Venn diagram showing differentially expressed genes in stage 1 and 3 eyestalks

Supplemental 4 - *C. quinquedens* MIH neuropeptide sequences

Supplemental 5 - *C. quinquedens* CHH neuropeptide sequences

Supplemental 6 - *C. quinquedens* eyestalk neuropeptide sequences

Chapter 3: Transcriptomic Analysis of vitellogenin and its potential cleaving enzymes from the hepatopancreas

Abstract

Vitellogenesis is the essential process for successful reproduction in ovipositing animals that involves the synthesis of yolk protein and its accumulation by developing oocytes. Decapod crustaceans utilize three main tissues for vitellogenesis and ovarian development: the hepatopancreas, hemolymph, and ovary. In most crab species, including the red deep-sea crab, *Chaceon quinquedens*, the hepatopancreas serves as the primary site of vitellogenin (the precursor of the yolk protein) production. This study used transcriptomic analysis to investigate genes (vitellogenin and endopeptidases) potentially significant to vitellogenesis to better understand the ovarian development of females. A hepatopancreas transcriptome for *C. quinquedens* females at ovarian stages 1 (early-vitellogenesis) and 3 (mid-vitellogenesis) was generated using next-generation RNA sequencing technologies. This hepatopancreas transcriptome produced 130,436 contigs and 76,043 genes. Two vitellogenin transcripts were pieced together to obtain the putative 2,570 amino acid vitellogenin protein. A stage-dependent vitellogenin expression was seen higher at stage 3 than at stage 1. In addition, three subtilisin-like endopeptidases for cleaving vitellogenin into subunits were identified: trypsin-like serine protease, furin, and proprotein convertase subtilisin/Kexin7. This study provides the first transcriptomic resource for *C. quinquedens* hepatopancreas and would enhance our understanding of the molecular mechanisms underlying vitellogenesis and ovarian development.

1. Introduction

Vitellogenesis is an essential process for the successful completion **of** reproduction in all ovipositing animals. In crustaceans, vitellogenin, the precursor of the main yolk protein, vitellin, is a large protein with just over 2500 amino acids. In decapods**,** the hepatopancreas and ovary are two major organs synthesizing the majority of vitellogenin (Jimenez-Gutierrez et al., 2019; Thongda et al., 2015). Vitellogenin produced in the hepatopancreas must be transported through the hemolymph to the oocytes where it will be absorbed via receptor-mediated endocytosis (Meusy and Payen, 1988; Zmora et al., 2007). However, this is not before it experiences its first cleavage at R-X-X-R and R-X-X-K motifs. Subtilisin-like endopeptidases are responsible for the cleavage of these motifs found throughout the vitellogenin protein (Tseng et al., 2001).

For over five decades, the red deep-sea crab, *Chaceon quinquedens* has inhabited depths ranging from 200 - 1800 meters and has supported a small commercial fishery in the western Atlantic Ocean(Chute et al., 2008; Haefner and Musick, 1974; Steimle et al., 2001; Weinberg and Keith, 2003). The harvest is driven by males larger than 94 mm carapace length (Chute et al., 2008), while females are prohibited from harvesting. With a recent change in the catch size limit, the fisheries based on cold-water species, like the red deep-sea crabs, especially those with slow growth rates, must rely on effective management strategies for sustainable harvest and population structure using input from the species' physiology. At present, the red deep-sea crab fishery lacks sufficient biological, growth, mortality, and reproduction data required to enhance its management plans.

Like other decapod species, female *C. quinquedens* reach sexual maturity, which is achieved at the puberty molt. Morphological features related to adult females of the genus *Chaceon* have been studied extensively (Attrill et al., 1991; Delgado and Defeo, 2004;

Fernández-Vergaz et al., 2000; Ferreira et al., 2016; Melville-Smith, 1988; Pinho et al., 2001; Tuset et al., 2011). An in-depth investigation into vitellogenesis in females of the genus *Chaceon* has yet to be undertaken. The completion of ovarian development in *C. quinquedens* includes 5 distinctive ovarian stages (Haefner, 1977; Martínez-Rivera and Stevens, 2020). There is currently no information on specific molecular reproductive activities occurring throughout each stage. Reproduction is influenced by internal factors, such as allocation of energy, metabolism, neuropeptides, and growth; as well as external factors such as fluctuations in temperature, salinity, oxygen levels, and food supply.

Seasonal changes affect all physiological processes in aquatic animals including vitellogenesis. Warm water temperatures can decrease the time needed to complete vitellogenesis for species such as the blue crab, *Callinectes sapidus* (Thongda et al., 2015; Zmora et al., 2007), and the freshwater prawn, *Macrobrachium occidentale* (Hernández-Sandoval et al., 2018). Conversely, female *C. quinquedens* and other members of its genus reside in deep cold waters, not affected by changes in seasons. Cold waters slow down growth and increase the time needed for *Chaceon* females to complete vitellogenesis and reproductive cycles (Biscoito et al., 2015; López-Abellán et al., 2002; Pezzuto and Sant' Ana, 2009). However, little is known about vitellogenesis in cold-water species like *C. quinquedens*. Knowledge of physiological processes such as vitellogenesis in *C. quinquedens* is necessary to understand how the animals regulate ovarian development, which could be essential for establishing sustainable fishery management plans.

Next-generation sequencing (NGS) has become an essential tool to investigate non-model organisms such as *C. quinquedens*. Transcriptomes generated by NGS technologies have been used to study physiological processes like reproductive activities. In the field of crustacean

reproduction, NGS was also employed crustaceans such as *Scylla paramamosain* (Bao et al., 2015; Gao et al., 2014), *Eriochier sinensis* (He et al., 2012; X. Liu et al., 2019; Su et al., 2020), *Macrobrachium nipponense* (Jiang et al., 2019; Qiao et al., 2017; Zhang et al., 2021), *Portunus trituberculatus* (M. Liu et al., 2019; Meng et al., 2015; Wang et al., 2018; Yang et al., 2015), *Litopenaeus vannamei* (Wang et al., 2019), *Nephrops norvegicus* (Rotllant et al., 2018, 2017), *Sagmariasus verreauxi* (Ventura et al., 2014), and *Cherax quadricarinatus* (Nguyen et al., 2016).

Herein, this study aimed to examine the specific genes involved in vitellogenesis: vitellogenin and endopeptidases in female *C. quinquedens*. RNA-sequencing technology generated a hepatopancreas transcriptome from females at ovarian stages 1 and 3. The hepatopancreas transcriptome contained complete the full-length vitellogenin sequence and identified subtilisin-like endopeptidases. The relative abundance of vitellogenin and candidate subtilisin-like endopeptidases was estimated from this transcriptome. This work serves as one of the first transcriptomic resources for *C. quinquedens*.

2. Materials and Methods

2.1 Animals

Female *C. quinquedens* were collected from the Mid-Atlantic Bight region of the United States. Females from ovarian developmental stage 1 (immature) and stage 3 (advanced) (Haefner, 1977; Martínez-Rivera et al., 2020) were collected in the summer of 2014 onboard the F/V Hannah Boden. Hepatopancreas tissue from females was dissected and placed in a 1.5 mL microcentrifuge tube containing 500 µl of RNA*later*® Solution (Ambion®). Upon arriving

Institute of Marine and Environmental Technology (IMET) samples were stored at -80°C until further processing.

2.2 De novo assembly and vitellogenin mining

The concentration of the total RNA extracted from hepatopancreas tissues at ovarian stages 1 and 3 (n = 3/stage) was estimated using a Nanodrop spectrometer. The RNA from three females at each stage with an equal amount per animal were pooled and submitted to Macrogen (www.macrogenusa.com) for library preparation and paired-end 150 base sequencing on the Illumina HiSeq 2500 platform.

The *de novo* assembly software Trinity (v. 2.8.6) was used to assemble paired-read sequences using the default parameters (including -trimmomatic to clean reads and no read normalization) (Grabherr et al., 2011; Haas et al., 2013). The completeness of the assembly was evaluated using the Benchmarking Universal Single-Copy Orthologs (BUSCO) approach by aligning to the arthropod reference set (Simão et al., 2015). Expression levels of the transcripts in the assemblies were estimated by remapping the high-quality reads on both transcriptomes separately with RNA-Seq by Expectation-Maximization (Robinson et al., 2010, Li and Dewey, 2011). RSEM data for both stages were further analyzed to determine the most highly expressed. Additionally, differential expression using edgeR at p-value (0.05) and log fold change >2.

2.3 Transcriptome Mining for Vitellogenin and Enzymes

A well-established workflow was used to identify putative vitellogenin protein sequence and subtilisin-like endopeptidases in hepatopancreas transcriptome (Christie, 2016, 2015, 2014; Christie et al., 2017, 2008; Christie and Pascual, 2016; Gard et al., 2009; Ma et al., 2010).

Vitellogenin sequences from decapod species were collected and placed in a text file using NCBIs efetch utility a command-line base (Supplemental data 1). These sequences were then BLASTed against the hepatopancreas assembly using an e-value cut-off 1e-05. The same process was followed to mine for the subtilisin-like endopeptidases (Supplemental data 2). Transcript per million (TPM) values >1 for vitellogenin and the subtilisin-like endopeptidases reported were extracted from the RSEM data to examine differences between ovarian stages.

Candidate vitellogenin and subtilisin-like endopeptidase nucleotide sequences were then compared to NCBI-GenBank database using BLASTX (https://blast.ncbi.nlm.nih.gov/). Contigs detected as vitellogenin, and subtilisin-like endopeptidase was translated using ExPASy Translate (web.expasy.org/translate) and signal peptides were identified with SignalP v.5.0 Server (http://www.cbs.dtu.dk/services/SignalP/). NCBI's Conserved Domain search tool (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), was used to identify all domains present in *C. quinquedens* vitellogenin. Phylogenetic analyses began with the alignment of selected decapod vitellogenin sequences using MAFFT, trimmed with GBLOCKS, and analyzed using PhyML with 100 bootstrap replicates as implemented by the phylogeny.fr website (https://www.phylogeny.fr).

3. Results

3.1 De novo assembly of C. quinquedens hepatopancreas transcriptome

Illumina sequencing on the HiSeq 2500 platform produced a total of 142 million raw reads from females at ovarian developmental stages 1 and 3 with 79 and 63 million read pairs, respectively. The assembly was aligned to the 1066 genes in the arthropod BUSCO reference and was 92% complete while 38.3% of the contigs were duplicated (Table 5) (C:92.4%[S:54.1%,

D:38.3%],F:4.1%,M:3.5%, n:1066). Alignment of reads back to the assembled transcriptome showed 85.55% of stage 1 and 85.96% of stage 3 reads were concordantly mapped. Comparing isoforms with TPM values greater than 1, 11,921 were unique to stage 1 and 8,006 were unique to stage 3 (Fig. 3.1). Together the two stages shared 43,443 isoforms (Fig. 3.1). Based on edgeR analysis, 10,465 of genes had greater than 2-fold expression differences between the two ovarian stages (Fig. 3.2).

3.2 Characterization of Vitellogenin

To obtain the complete vitellogenin amino sequence, the hepatopancreas transcriptome was mined for transcripts that proved to be portions of the hormone (Supplemental Data 3). *C. quinquedens* vitellogenin was pieced together from two transcripts from the same trinity graph component that overlapped by over 1700 amino acids, TRINITY_DN2_c0_g1 and TRINITY_DN2_c0_g2. When aligned, the complete open reading frame encodes a 2,570 aa protein (Fig. 3.3). There are three isoforms from the transcript TRINITY_DN2_c0_g2 (TRINITY_DN2_c0_g2_i4, TRINITY_DN2_c0_g2_i6, and TRINITY_DN2_c0_g2_i8) on the same graph component that represented fragments of the 3' end of vitellogenin. Of these were shorter versions of TRINITY_DN2_c0_g2 (982 aa for transcripts TRINITY_DN2_c0_g2_i4 and TRINITY_DN2_c0_g2_i6; with TRINITY_DN2_c0_g2_i8 containing 789 aa). Overall, there were 20 amino acid differences in the aligned region and a single consensus translation was made using the majority rule.

To further characterize *C. quinquedens* vitellogenin, the sequence was checked for the presence of a signal peptide which was found to be the first 18 amino acids followed by a 2,552 amino acid protein. Three conserved domains common to crustacean vitellogenin were identified (Fig. 3.3). The vitellogenin_N domain is 546 aa and encodes residues 41 through 587. This

sequence aligned 100% to the vitellogenin_N cloned (*Green et al. 2022 under review*). The next conserved region was the domain of unknown function 1943 (DUF1943), the 302 aa represent residues 620 through 922. Like most crab vitellogenin, the von Wilderbrand factor type D is present in the C-terminus (VWD) and its 141 aa are found between amino acids 2,337 to 2,478. When creating a phylogenetic tree, *C. quinquedens* vitellogenin is a part of the clade that includes other crab species. Its closest identity is to *C. sapidus* at 87% and *S. paramamosain* at 78% (Fig. 3.4).

3.3 Identifying enzymes potentially cleaving the vitellogenin into subunits

Crustacean vitellogenin amino acid sequences are known for containing multiple R-X-X-R motifs. These motifs are cleaved by subtilisin-like endopeptidases (Chan et al., 2005; Mak et al., 2005; Tiu et al., 2009; Tseng et al., 2001). This study selected three enzymes belonging to the subtilisin-like endopeptidase family: trypsin-like serine protease, furin, and proprotein convertase subtilisin/kexin type 7. The *C. quinquedens* vitellogenin has seven such R-X-X-R and R-X-X-K motifs (RVQR 119-122; RLAK 521-524; RERR 729-732; RIRR 949-952; RRIK 1443-1446; RAIR 1679-1682; and RISR 1886 – 1889) with the potential to be cleaved by any of the previously stated enzymes.

3.4 Differential expression analysis of vitellogenin and subtilisin-like endopeptidases

Differentially expressed transcripts in the hepatopancreas between stages 1 and 3 were calculated using TPM. Vitellogenin TPM values were lower at stage 1 (2,875) and then increased at stage 3 (924,475) (Fig. 3.5). Candidates potentially making vitellogenin's first cleavage,

trypsin-like serine protease expression increases from stage 1 to 3; furin expression is higher at stage 1 and decreases at stage 3; and proprotein convertase subtilisin/kexin type 7 expression increases from stage 1 to 3.

4. Discussion

In this chapter, NGS was employed to generate a hepatopancreas transcriptome from females at ovarian stages 1 and 3. The physiological process of vitellogenesis in adult female decapods residing in shallow waters has been the topic of several studies. However, the opposite is true for decapods inhabiting depths >200 m, where vitellogenesis is a poorly understood process. We created the first *de novo* assembled transcriptome from the hepatopancreas of female *C. quinquedens*. The *C. quinquedens* transcriptome is valid, showing high BUSCO scores (92.4%) compared to other arthropod genes.

Crustacean reproduction largely depends on ovarian development and maturation. Therefore, the onset of vitellogenesis is closely related to female sexual maturity. Two major tissues in female crustaceans: the hepatopancreas and ovary, are involved in the vitellogenin synthesis, where transcription and translation of vitellogenin occur. The hemolymph usually carries the protein vitellogenin from these two synthesis sites to the target site, the ovary, where it is uptake via receptor-mediated endocytosis.

In most crab species, the hepatopancreas serves as the primary site (>99%) of vitellogenin synthesis, as the precursor with ~250kDa, consisting of ~2500 amino acid residues (Thongda et al., 2015; Zmora et al., 2007). In shrimp, the hepatopancreas and ovary show an equal contribution of vitellogenin for ovarian development (Tsukimura, 2001). Upon the translation in the hepatopancreas, vitellogenin is subjected for the first cleavage prior to being

released into the hemolymph as two subunits: ~78 and ~180kDa. In the ovary, the larger subunit undergoes the second cleavage by subtilisin-like endopeptidase into ~80 and ~100kDa subunits. The cleavage processes of vitellogenin are common in all decapod crustaceans.

In decapod crustaceans, ovarian developments are often presented with vitellogenin transcripts and protein levels in the hemolymph. Vitellogenin transcripts accumulating in the hepatopancreas and ovary parallel ovarian development in all crustaceans. The translation rate seems to remain constant; vitellogenin levels in the hemolymph are also accumulated, reflecting the ovarian stage.

The duration of completing ovarian development for the spawning of the eggs depends on the species and their environmental conditions. The levels of vitellogenin in the hepatopancreas and hemolymph also differ by species. In warm-water species, like *C. sapidus*, the completion of vitellogenesis takes ~8 weeks from stage 1 to stage 4, reaching the peak levels of vitellogenin from ~ <100 ug to ~ 2000 ug/ml, respectively (Bembe et al., 2018; Zmora et al., 2007). On the other hand, cold water species seem to take a much more extended period for ovarian development, influencing vitellogenin levels (Haefner, 1977; Martinez-Rivera, 2018).

Investigations into reproductive genes and mechanisms using transcriptomic techniques in crustaceans have increased in recent years. Transcriptomic analysis in species such as *E. sinesis* (He et al., 2012; Jiang et al., 2009), *S. paramamosain* (Bao et al., 2015), *P. trituberculatus* (Meng et al., 2015), *Nephrops norvegicus* (Rotllant et al., 2018, 2017) and many others have revealed many key participants and pathways developing our understanding of the vitellogenesis process in female decapods. Our study identified the two transcripts that pieced together the complete 2,570 aa vitellogenin protein. Conserved domains in *C. quinquedens* vitellogenin are similar to the majority of other crabs containing a vitellogenin_N, DUF 1043,

and VWD (Jimenez-Gutierrez et al., 2019). The highly conserved vitellogenin_N domain from this sequence was in complete alignment (100%) with the partially sequenced isolated by Green et al. (*under review*). Several vitellogenin sequences from both the hepatopancreas and ovary have been identified in crustaceans (Jimenez-Gutierrez et al., 2019; Thongda et al., 2015). In female *C. quinquedens* the hepatopancreas is the major producer of vitellogenin producing ~1000 fold more than the ovary (Huang et al., 2020).

As expected, the hepatopancreas transcriptome TPM values (>1) for vitellogenin were higher in females at stage 3 compared to those at stage 1. The collection of stage 1 red deep-sea crab females is limited due to fishery regulations and depth limits. Transcript values of vitellogenin at this immature phase can aid vitellogenin expression from stages 2 through 5 reported by Green et al. (*in review*). The *C. quinquedens* vitellogenin expression also complements the work of Martinez-Rivera and Stevens (2020), noting the oocyte diameter that increases from 32 ± μm at stage 1 to 298 ± 132 μm at 3 due to the accumulation of vitellogenin. Vitellogenin expression increases in most decapods as ovarian development progresses (Mak et al., 2005) and is also observed in *C. quinquedens*.

Vitellogenin cleavage consensus R-X-X-R motifs are found in mammals, insects, and crustaceans (Chen and Raikhel, 1996; Xie et al., 2009). If *C. quinquedens* vitellogenin experiences its first cleavage by a subtilisin-like endopeptidase prior to the RERR motif (Arg^{729} – Arg^{732}) the resulting two subunits would measure 80 kDa and 207 kDa (Fig. 3.3). Several other decapods speculate the first cleavage to be between amino acids (AA) 710-738, including *Homarus americanus* AA728 – AA731 (Tiu et al., 2009), *L. vannamei* AA725 – AA728 (Raviv et al., 2006), *C. sapidus* AA728 – AA731 (Zmora et al., 2007), *Charybdis feriata* (Mak et al.,

2005). The *C. Quinquedens* vitellogenin sequence has seven R-X-X-R motifs present similar to within the range of other decapods (Mak et al., 2005).

Most crustacean studies mainly make note that R-X-X-R motifs are cleaved by subtilisin-like endopeptidases without specifically identifying candidates. The identity of candidates responsible for the two lipoprotein subunits produced in the fat body of insects has been studied (Chen and Raikhel, 1996; Smolenaars et al., 2007, 2005). Subtilisin-like endopeptidases with the potential to conduct this initial cleavage were searched in the transcriptome and three enzymes were chosen: furin, a trypsin-like serine protease, and proprotein convertase subtilisin/kexin type 7. All of the subtilisin-like endopeptidases were found to cleave the conserved motif in insects (Sathapondecha et al., 2017; Smolenaars et al., 2007). Trypsin-like serine protease and proprotein convertase subtilisin/kexin type 7 values increase between stages 1 and 3, which is the same trend for vitellogenin. These increases in values between each of these enzymes could be responsible for the cleavage of increased vitellogenin protein during stage 3.

5. Conclusion

We explored the transcriptome and identified the 2,570 aa *C. quinquedens* vitellogenin protein. Studies identifying specific subtilisin-like endopeptidases responsible for creating two subunits of vitellogenin in the hepatopancreas of crustaceans are extremely limited. Future studies should be conducted to determine whether these candidates are involved in the cleavage R-X-X-R sites within vitellogenin. This study provides new essential information on the reproductive biology of *C. quinquedens.* A significant influence on the conservation and sustainability of the *C. quinquedens* population is the understanding of the length of time needed to complete a full vitellogenic cycle. This addition is useful in continuing to find genes important to the reproduction of the species that can assist future fishery management plans.

References

Attrill, M.J., Hartnoll, R.G., Rice, A.L., 1991. Aspects of the biology of the deep-sea crab *Geryon trispinosus* from the porcupine seabight. Journal of the Marine Biological Association of the United Kingdom 71, 311–328. https://doi.org/10.1017/S0025315400051626

Bao, C., Yang, Y., Huang, H., Ye, H., 2015. Neuropeptides in the cerebral ganglia of the mud crab, *Scylla paramamosain*: transcriptomic analysis and expression profiles during vitellogenesis. Scientific Reports 5. https://doi.org/10.1038/srep17055

Bembe, S., Zmora, N., Williams, E., Place, A.R., Liang, D., Chung, J.S., 2018. Effects of temperature and photoperiod on hemolymph vitellogenin levels during spawning events of the blue crab, Callinectes sapidus, in captivity. Aquaculture Research 49, 2201–2209. https://doi.org/10.1111/are.13676

Biscoito, M., Freitas, M., Pajuelo, J.G., Triay-Portella, R., Santana, J.I., Costa, A.L., Delgado, J., González, J.A., 2015. Sex-structure, depth distribution, intermoult period and reproductive pattern of the deep-sea red crab *Chaceon affinis* (Brachyura, Geryonidae) in two populations in the north-eastern Atlantic. Deep-Sea Research Part I: Oceanographic Research Papers 95, 99–114. https://doi.org/10.1016/j.dsr.2014.10.010

Chan, S.M., Mak, A.S.C., Choi, C.L., Ma, T.H.T., Hui, J.H.L., Tiu, S.H.K., 2005. Vitellogenesis in the red crab, *Charybdis feriatus*: contributions from small vitellogenin transcripts (CfVg) and farnesoic acid stimulation of CfVg expression., in: Annals of the New York Academy of Sciences. New York Academy of Sciences, pp. 74–79. https://doi.org/10.1196/annals.1327.008

Chen, J.S., Raikhel, A.S., 1996. Subunit cleavage of mosquito pro-vitellogenin by a subtilisin-like convertase. Proceedings of the National Academy of Sciences of the United States of America 93, 6186–6190. https://doi.org/10.1073/pnas.93.12.6186

Christie, A.E., 2016. Expansion of the neuropeptidome of the globally invasive marine crab *Carcinus maenas*. General and Comparative Endocrinology 235, 150–169. https://doi.org/10.1016/j.ygcen.2016.05.013

Christie, A.E., 2015. Neuropeptide discovery in *Symphylella vulgaris* (Myriapoda, Symphyla): In silico prediction of the first myriapod peptidome. General and Comparative Endocrinology 223, 73–86. https://doi.org/10.1016/j.ygcen.2015.09.021

Christie, A.E., 2014. Prediction of the peptidomes of *Tigriopus californicus* and *Lepeophtheirus salmonis* (Copepoda, Crustacea). General and Comparative Endocrinology 201, 87–106. https://doi.org/10.1016/j.ygcen.2014.02.015

Christie, A.E., Cashman, C.R., Stevens, J.S., Smith, C.M., Beale, K.M., Stemmler, E.A., Greenwood, S.J., Towle, D.W., Dickinson, P.S., 2008. Identification and cardiotropic actions of brain/gut-derived tachykinin-related peptides (TRPs) from the American lobster *Homarus americanus*. Peptides 29, 1909–1918. https://doi.org/10.1016/j.peptides.2008.07.010

Christie, A.E., Pascual, M.G., 2016. Peptidergic signaling in the crab Cancer borealis: Tapping the power of transcriptomics for neuropeptidome expansion. General and Comparative Endocrinology 237, 53–67. https://doi.org/10.1016/j.ygcen.2016.08.002

Christie, A.E., Roncalli, V., Cieslak, M.C., Pascual, M.G., Yu, A., Lameyer, T.J., Stanhope, M.E., Dickinson, P.S., 2017. Prediction of a neuropeptidome for the eyestalk ganglia of the lobster *Homarus americanus* using a tissue-specific de novo assembled transcriptome. General and Comparative Endocrinology 243, 96–119. https://doi.org/10.1016/j.ygcen.2016.11.001

Chute, A., Jacobson, L., Rago, P., MacCall, A., 2008. Deep Sea red crab. Northeast Data Poor Stocks Working Group Meeting 1–18.

Delgado, E., Defeo, O., 2004. Sexual maturity in females of deep-sea red crab *Chaceon notialis* (Brachyura, Geryonidae) in the southwestern Atlantic Ocean. Invertebrate Reproduction and Development 46, 55–62.

Elner, R.W., Koshio, S., Hurley, G.V., 1987. Mating behavior of the deep-sea red crab, *Geryon quinquedens* Smith (Decapoda, Brachyura, Geryonidae). Crustaceana 52, 194–201.

Fernández-Vergaz, V., López Abellán, L.J., Balguerías, E., 2000. Morphometric, functional and sexual maturity of the deep-sea red crab *Chaceon affinis* inhabiting Canary Island waters: chronology of maturation. Marine Ecology Progress Series 204, 169–178. https://doi.org/10.3354/meps204169

Ferreira, R.C.P., Nunes, D.M., Shinozaki-Mendes, R.A., Pires, A.M.A., Hazin, F.H.V., 2016. First record and preliminary information on the biology of the deep-sea African crab,

Chaceon gordonae (Ingle, 1985) (Brachyura: Geryonidae) in Saint Peter and Saint Paul Archipelago, Brazil. Latin American Journal of Aquatic Research 44, 392–400. https://doi.org/10.3856/vol44-issue2-fulltext-20

Gao, J., Wang, X., Zou, Z., Jia, X., Wang, Y., Zhang, Z., 2014. Transcriptome analysis of the differences in gene expression between testis and ovary in green mud crab (*Scylla paramamosain*). BMC Genomics 15. https://doi.org/10.1186/1471-2164-15-585

Gard, A.L., Lenz, P.H., Shaw, J.R., Christie, A.E., 2009. Identification of putative peptide paracrines/hormones in the water flea Daphnia pulex (Crustacea; Branchiopoda; Cladocera) using transcriptomics and immunohistochemistry. General and Comparative Endocrinology 160, 271–287. https://doi.org/10.1016/j.ygcen.2008.12.014

Grabherr, M.G., Haas, B.J., Yassour, M., Levin, J.Z., Thompson, D.A., Amit, I., Adiconis, X., Fan, L., Raychowdhury, R., Zeng, Q., Chen, Z., Mauceli, E., Hacohen, N., Gnirke, A., Rhind, N., di Palma, F., Birren, B.W., Nusbaum, C., Lindblad-Toh, K., Friedman, N., Regev, A., 2011. Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nature Biotechnology 29, 644–652. https://doi.org/10.1038/nbt.1883

Haas, B.J., Papanicolaou, A., Yassour, M., Grabherr, M., Blood, P.D., Bowden, J., Couger, M.B., Eccles, D., Li, B., Lieber, M., Macmanes, M.D., Ott, M., Orvis, J., Pochet, N., Strozzi, F., Weeks, N., Westerman, R., William, T., Dewey, C.N., Henschel, R., Leduc, R.D., Friedman, N., Regev, A., 2013. De novo transcript sequence reconstruction from RNA-seq using the Trinity platform for reference generation and analysis. Nature Protocols 8, 1494–1512. https://doi.org/10.1038/nprot.2013.084

Haefner, P.A., 1977. Reproductive biology of the female Deep-Sea Red Crab, *Geryon quinquedens*, from the Chesapeake Bight. Fishery Bulletin 75, 91–102.

Haefner, P.A., Musick, J.A., 1974. Observations on Distribution and Abundance of Red Crabs in Norfolk Canyon and Adjacent Continental Slope. Marine Fisheries Review 36, 31–34.

He, L., Wang, Q., Jin, X., Wang, Ying, Chen, L., Liu, L., Wang, Yang, 2012. Transcriptome profiling of testis during sexual maturation stages in *Eriocheir sinensis* using Illumina sequencing. PloS ONE 7. https://doi.org/10.1371/journal.pone.0033735

Hernández-Sandoval, P., Díaz-Herrera, F., Díaz-Gaxiola, J.M., Martínez-Valenzuela, C., García-Guerrero, M., 2018. Effect of temperature on growth, survival, thermal behavior, and critical thermal maximum in the juveniles of *Macrobrachium occidentale* (Holthuis, 1950)

(Decapoda: Caridea: Palaemonidae) from Mexico. Journal of Crustacean Biology 38, 483–488. https://doi.org/10.1093/jcbiol/ruy024

Huang, X., Green, S., Sook Chung, J., 2020. The presence of an insulin-like peptide-binding protein (ILPBP) in the ovary and its involvement in the ovarian development of the red deep-sea crab, *Chaceon quinquedens*. General and Comparative Endocrinology 301, 113653. https://doi.org/10.1016/j.ygcen.2020.113653

Jiang, H., Yin, Y., Zhang, X., Hu, S., Wang, Q., 2009. Chasing relationships between nutrition and reproduction: A comparative transcriptome analysis of hepatopancreas and testis from *Eriocheir sinensis*. Comparative Biochemistry and Physiology – Part D: Genomics and Proteomics 4, 227–234. https://doi.org/10.1016/j.cbd.2009.05.001

Jiang, Q., Min, Y., Yang, H., Wan, W., Zhang, X., 2019. De novo transcriptome analysis of eyestalk reveals ovarian maturation related genes in *Macrobrachium rosenbergii*. Aquaculture 505, 280–288. https://doi.org/10.1016/j.aquaculture.2019.02.073

Jimenez-Gutierrez, S., Cadena-Caballero, C.E., Barrios-Hernandez, C., Perez-Gonzalez, R., Martinez-Perez, F., Jimenez-Gutierrez, L.R., 2019. Crustacean vitellogenin: A systematic and experimental analysis of their genes, genomes, mRNAs and proteins; and perspective to Next Generation Sequencing. Crustaceana 92, 1169–1205. https://doi.org/10.1163/15685403-00003930

Liu, M., Pan, J., Dong, Z., Cheng, Y., Gong, J., Wu, X., 2019. Comparative transcriptome reveals the potential modulation mechanisms of estradiol affecting ovarian development of female *Portunus trituberculatus*. PloS ONE 14, 1–21. https://doi.org/10.1371/journal.pone.0226698

Liu, X., Ma, K.Y., Liu, Z.Q., Feng, J. bin, Ye, B.Q., Qiu, G.F., 2019. Transcriptome analysis of the brain of the Chinese mitten crab, *Eriocheir sinensis*, for neuropeptide abundance profiles during ovarian development. Animal Reproduction Science 201, 63–70. https://doi.org/10.1016/j.anireprosci.2018.12.010

López-Abellán, L.J., Balguerías, E., Fernández-Vergaz, V., 2002. Life history characteristics of the deep-sea crab *Chaceon affinis* population off Tenerife (Canary Islands). Fisheries Research 58, 231–239. https://doi.org/10.1016/S0165-7836(01)00384-8

Ma, M., Gard, A.L., Xiang, F., Wang, J., Davoodian, N., Lenz, P.H., Malecha, S.R., Christie, A.E., Li, L., 2010. Combining in silico transcriptome mining and biological mass

spectrometry for neuropeptide discovery in the Pacific white shrimp Litopenaeus vannamei. Peptides 31, 27–43. https://doi.org/10.1016/j.peptides.2009.10.007

Mak, A.S.C., Choi, C.L., Tiu, S.H.K., Hui, J.H.L., He, J.G., Tobe, S.S., Chan, S.M., 2005. Vitellogenesis in the red crab *Charybdis feriatus*: hepatopancreas-specific expression and farnesoic acid stimulation of vitellogenin gene expression. Molecular Reproduction and Development 70, 288–300. https://doi.org/10.1002/mrd.20213

Martinez-Rivera, S., 2018. Reproductive biology of the female red deep-sea crab, *Chaceon quinquedens* (Smith, 1879), in the Mid-Atlantic Bight.

Martínez-Rivera, S., Long, W.C., Stevens, B.G., 2020. Physiological and behavioral sexual maturity of female red deep-sea crabs *Chaceon quinquedens* (Smith, 1879) (Decapoda: Brachyura: Geryonidae) in the Mid-Atlantic Bight. Journal of Crustacean Biology 40, 330–340. https://doi.org/10.1093/jcbiol/ruaa007

Martínez-Rivera, S., Stevens, B.G., 2020. Embryonic development and fecundity of the red deep-sea crab *Chaceon quinquedens* (Smith, 1879) (Decapoda: Brachyura: Geryonidae) in the Mid-Atlantic Bight determined by image analysis. Journal of Crustacean Biology 40, 230–236. https://doi.org/10.1093/JCBIOL/RUAA017

Melville-Smith, R., 1988. The Commercial Fishery for and Population Dynamics of Red Crab Geryon maritae off Southwest Africa, 1976-1986. South African Journal of Marine Sciences 5, 79–95.

Meng, X.L., Liu, P., Jia, F.L., Li, J., Gao, B.Q., 2015. De novo transcriptome analysis of *Portunus trituberculatus* ovary and testis by RNA-Seq: Identification of genes involved in gonadal development. PloS ONE 10. https://doi.org/10.1371/journal.pone.0128659

Meusy, J.-J., Payen, G.G., 1988. Female reproduction in Malacostracan crustacea regulation. Zoological Science 5, 217–265.

Nguyen, T.V., Cummins, S.F., Elizur, A., Ventura, T., 2016. Transcriptomic characterization and curation of candidate neuropeptides regulating reproduction in the eyestalk ganglia of the Australian crayfish, *Cherax quadricarinatus*. Scientific Reports 6, 1–19. https://doi.org/10.1038/srep38658

Pezzuto, P.R., Sant' Ana, R., 2009. Sexual maturity of the deep-sea royal crab *Chaceon ramosae* Manning, Tavares & Albuquerque, 1989 (Brachyura: Geryonidae) in southern Brazil. Latin

American Journal of Aquatic Research 37, 285–296. https://doi.org/10.3856/vol37-issue3-fulltext-2

Pinho, M.R., Gonçalves, J.M., Martins, H.R., Menezes, G.M., 2001. Some aspects of the biology of the deep-water crab, *Chaceon affinis* (Milne-Edwards and Bouvier, 1894) off the Azores. Fisheries Research 51, 283–295.

Qiao, H., Fu, H., Xiong, Y., Jiang, S., Zhang, W., Sun, S., Jin, S., Gong, Y., Wang, Y., Shan, D., Li, F., Wu, Y., 2017. Molecular insights into reproduction regulation of female Oriental River prawns *Macrobrachium nipponense* through comparative transcriptomic analysis. Scientific Reports 7, 1–11. https://doi.org/10.1038/s41598-017-10439-2

Raviv, S., Parnes, S., Segall, C., Davis, C., Sagi, A., 2006. Complete sequence of Litopenaeus vannamei (Crustacea: Decapoda) vitellogenin cDNA and its expression in endocrinologically induced sub-adult females. General and Comparative Endocrinology 145, 39–50. https://doi.org/10.1016/j.ygcen.2005.06.009

Rotllant, G., Nguyen, T.V., Hurwood, D., Sbragaglia, V., Ventura, T., Company, J.B., Joly, S., Elizur, A., Mather, P.B., 2018. Evaluation of genes involved in Norway lobster (*Nephrops norvegicus*) female sexual maturation using transcriptomic analysis. Hydrobiologia 825, 137–158. https://doi.org/10.1007/s10750-018-3521-3

Rotllant, G., Nguyen, T.V., Sbragaglia, V., Rahi, L., Dudley, K.J., Hurwood, D., Ventura, T., Company, J.B., Chand, V., Aguzzi, J., Mather, P.B., 2017. Sex and tissue specific gene expression patterns identified following de novo transcriptomic analysis of the Norway lobster, *Nephrops norvegicus*. BMC Genomics 18, 1–14. https://doi.org/10.1186/s12864-017-3981-2

Sathapondecha, P., Panyim, S., Udomkit, A., 2017. In vitro study of a putative role of gonad-inhibiting hormone in oocyte growth stimulation in Penaeus monodon. Aquaculture Research 48, 5846–5853. https://doi.org/10.1111/are.13407

Simão, F.A., Waterhouse, R.M., Ioannidis, P., Kriventseva, E. v., Zdobnov, E.M., 2015. BUSCO: Assessing genome assembly and annotation completeness with single-copy orthologs. Bioinformatics 31, 3210–3212. https://doi.org/10.1093/bioinformatics/btv351

Smolenaars, M.M.W., Kasperaitis, M.A.M., Richardson, P.E., Rodenburg, K.W., van der Horst, D.J., 2005. Biosynthesis and secretion of insect lipoprotein: Involvement of furin in cleavage

of the apoB homolog, apolipophorin-II/I. Journal of Lipid Research 46, 412–421. https://doi.org/10.1194/jlr.M400374-JLR200

Smolenaars, M.M.W., Madsen, O., Rodenburg, K.W., van der Horst, D.J., 2007. Molecular diversity and evolution of the large lipid transfer protein superfamily. Journal of Lipid Research 48, 489–502. https://doi.org/10.1194/jlr.R600028-JLR200

Steimle, F.W., Zetlin, C.A., Chang, S., 2001. Red deepsea crab, *Chaceon (Geryon) quinquedens*, life history and habitat characteristics., NOAA Tech. Memo. NMFS-NE-163.

Stevens, B.G., Guida, V., 2016. Depth and temperature distribution, morphometrics, and sex ratios of red deepsea crab (*Chaceon quinquedens*) at 4 sampling sites in the Mid-Atlantic Bight. Fishery Bulletin 114, 343–359. https://doi.org/10.7755/FB.114.3.7

Su, Y., Guo, Q., Gong, J., Cheng, Y., Wu, X., 2020. Functional expression patterns of four 88cdysteroids receptor isoforms indicate their different functions during vitellogenesis of Chinese mitten crab, *Eriocheir sinensis*. Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology 248. https://doi.org/10.1016/j.cbpa.2020.110754

Thongda, W., Chung, J.S., Tsutsui, N., Zmora, N., Katenta, A., 2015. Seasonal variations in reproductive activity of the blue crab, *Callinectes sapidus*: Vitellogenin expression and levels of vitellogenin in the hemolymph during ovarian development. Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology 179, 35–43. https://doi.org/10.1016/j.cbpa.2014.08.019

Tiu, S.H.K., Hui, H.L., Tsukimura, B., Tobe, S.S., He, J.G., Chan, S.M., 2009. Cloning and expression study of the lobster (*Homarus americanus*) vitellogenin: conservation in gene structure among decapods. General and Comparative Endocrinology 160, 36–46. https://doi.org/10.1016/j.ygcen.2008.10.014

Tseng, D.-Y., Chen, Y.-N., Kou, G.-H., Lo, C.-F., Kuo, C.-M., 2001. Hepatopancreas is the extraovarian site of vitellogenin synthesis in black tiger shrimp, *Penaeus monodon*. Comparative Biochemistry and Physiology Part A 129, 909–917.

Tsukimura, B., 2001. Crustacean vitellogenesis: its role in oocyte development 1. American Zoologist 41, 465–476. https://doi.org/10.1668/0003-1569(2001)041[0465:CVIRIO]2.0.CO;2

Tuset, V.M., Espinosa, D.I., García-Mederos, A., Santana, J.I., González, J.A., 2011. Egg development and fecundity estimation in deep-sea red crab, *Chaceon affinis* (Geryonidae),

off the Canary Islands (NE Atlantic). Fisheries Research 109, 373–378. https://doi.org/10.1016/j.fishres.2011.02.021

Ventura, T., Cummins, S.F., Fitzgibbon, Q., Battaglene, S., Elizur, A., 2014. Analysis of the central nervous system transcriptome of the Eastern rock lobster *Sagmariasus verreauxi* reveals its putative neuropeptidome. PloS ONE 9. https://doi.org/10.1371/journal.pone.0097323

Wang, Z., Luan, S., Meng, X., Cao, B., Luo, K., Kong, J., 2019. Comparative transcriptomic characterization of the eyestalk in Pacific white shrimp (*Litopenaeus vannamei*) during ovarian maturation. General and Comparative Endocrinology 274, 60–72. https://doi.org/10.1016/j.ygcen.2019.01.002

Wang, Z., Sun, L., Guan, W., Zhou, C., Tang, B., Cheng, Y., Huang, J., Xuan, F., 2018. De novo transcriptome sequencing and analysis of male and female swimming crab (*Portunus trituberculatus*) reproductive systems during mating embrace (stage II). BMC Genetics 19. https://doi.org/10.1186/s12863-017-0592-5

Weinberg, J.R., Keith, C., 2003. Population size-structure of harvested deep-sea red crabs (*Chaceon quinquedens*) in the northwest Atlantic Ocean. Crustaceana 79, 819–833.

Xie, S., Sun, L., Liu, F., Dong, B., 2009. Molecular characterization and mRNA transcript profile of vitellogenin in Chinese shrimp, *Fenneropenaeus chinensis*. Molecular Biology Reports 36, 389–397. https://doi.org/10.1007/s11033-007-9192-1

Yang, Y., Wang, J., Han, T., Liu, T., Wang, C., Xiao, J., Mu, C., Li, R., Yu, F., Shi, H., 2015. Ovarian transcriptome analysis of *Portunus trituberculatus* provides insights into genes expressed during phase III and IV development. PloS ONE 10. https://doi.org/10.1371/journal.pone.0138862

Zhang, Y., Jiang, S., Qiao, H., Xiong, Y., Fu, H., Zhang, W., Gong, Y., Jin, S., Wu, Y., 2021. Transcriptome analysis of five ovarian stages reveals gonad maturation in female *Macrobrachium nipponense*. BMC Genomics 22. https://doi.org/10.1186/s12864-021-07737-5

Zmora, N., Trant, J., Chan, S.M., Chung, J.S., 2007. Vitellogenin and its messenger RNA during ovarian development in the female blue crab, *Callinectes sapidus*: gene expression, synthesis, transport, and cleavage. Biology of Reproduction 77, 138–146. https://doi.org/10.1095/biolreprod.106.055483

Tables:

Table 5. Statistics

Total reads Hepatopancreas Stage 1	79,395,216 (39,697,608 paired)
Total reads Hepatopancreas Stage 3	62,995,038 (31,497,519 paired)
Total	142,390,254
Percent GC	45.36
Isoforms	
Number of transcripts	128,141
Average contigs	871
N50	1602
Total assembled bases	111,647,056
BUSCO	C:92.4%[S:54.1%,D:38.3%],F:4.1%,M:3.5%]
Genes	
Total number of genes	76,043
Average gene size (bp)	739
N50	1259
Total assembled bases	56,159,164

Figures:

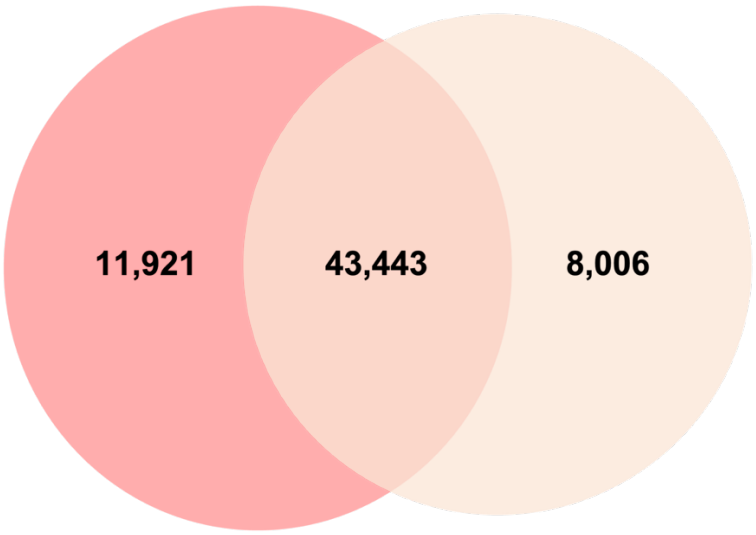


Fig. 3.1. Venn diagram of the differentially expressed. Venn diagram showing the number of significantly changing transcripts unique to stage 1 (pink circle), and unique to stage 3 (beige circle), and those that showed significant shifts in both stages (middle).

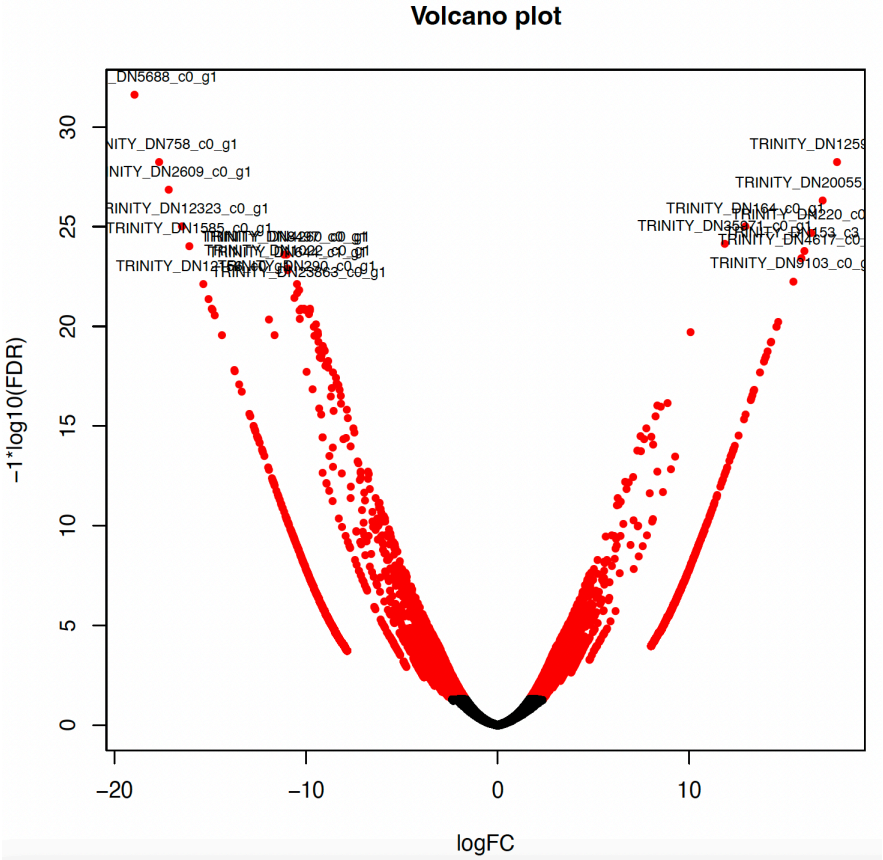


Fig. 3.2. Volcano Plot of stage 1 and 3 hepatopancreas transcriptome. 'Volcano plot' of statistical significance against fold-change between stage 1 and 3 females. The log2 fold-change indicates the mean expression level for each gene. Each dot represents one gene.

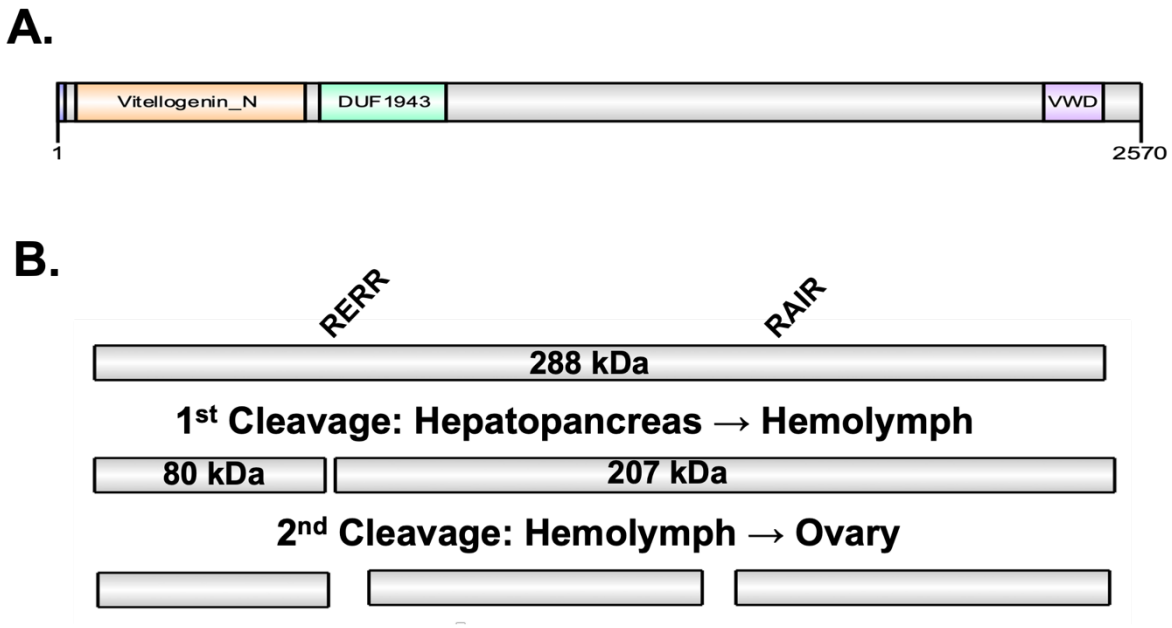


Fig. 3.3. Schematic diagram of *C. quinquedens* vitellogenin. (**A**) Schematic representation of the vitellogenin protein with conserved regions listed as follows signal peptide (blue); vitellogenin_N (orange); Domain of Unknown Function 1943 (green); von Wilderbrand factor type D (purple). (**B)** Schematic of vitellogenin of the proposed cleavage sites in *C. quinquedens* hepatopancreas (RERR) and hemolymph (RAIR).

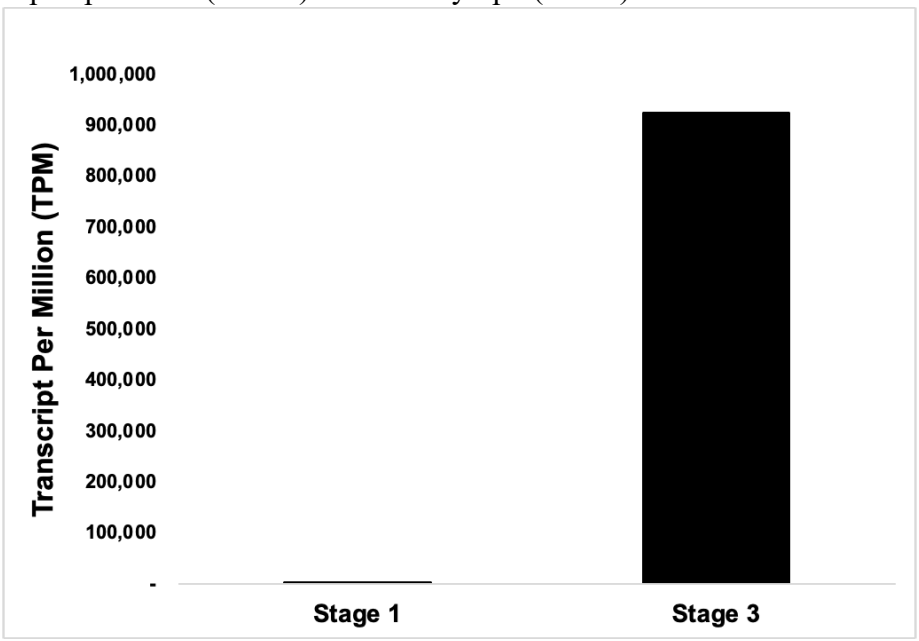


Fig. 3.5. Vitellogenin expression in *C. quinquedens*. *C. quinquedens* vitellogenin transcript abundance in the hepatopancreas during stages 1 and 3.

Chapter 4: Development and validation of novel microsatellite markers in the red deep-sea crab, *Chaceon quinquedens*

Abstract

The red deep-sea crab, *Chaceon quinquedens*, is a cold-water decapod found along the continental shelf and slope of the eastern United States. Since the early 1970s, the red deep-sea crab has supported a productive commercial fishery along the eastern U.S. (from the Gulf of Maine to Cape Hatteras, NC). Currently, there is very limited data on the genetic structure of red deep-sea crab populations, i.e., whether distinct stocks exist and over what spatial scales, and no nuclear genetic markers specific to *C. quinquedens* have been developed. This study aimed to develop microsatellite markers for population genetic analysis of *C. quinquedens* using Illumina sequencing of genomic DNA and a bioinformatic pipeline to identify repeat motifs, followed by PCR-based lab testing and validation of markers. Over 14 million paired-end reads were generated from sequencing hemocyte samples for six *C. quinquedens* individuals, which were assembled into 263,435 contigs >80bp. In total over 37,000 microsatellites were identified (2240 perfect repeats), from which 122 markers were considered for initial PCR testing with a limited number of crabs (n = 7). Fourteen markers were determined to be polymorphic and scorable, and three of these markers were chosen for a preliminary investigation of the genetic structure of *C. quinquedens* populations within three management areas (n = 88). An average of 21 alleles were identified across the three loci and the mean expected (H_e) and observed heterozygosity (H_o) were 0.879 and 1, respectively, thus indicating high levels of genetic diversity for these three populations or management areas. Analysis of genetic differentiation among populations revealed a mean F_{ST} value of 0.035, which is indicative of subtle but significant genetic structure among populations. Overall, the development of 14 novel, polymorphic microsatellite markers for the red deep-sea crab provides much-needed genetic resources for the management of the

species and should facilitate future, larger analyses of genetic diversity and stock structure in deep-sea red crabs.

1. Introduction

The Atlantic red deep-sea crab, *Chaceon quinquedens,* inhabits the Atlantic Ocean coastal waters from Nova Scotia to the Gulf of Mexico at depths ranging from 200 - 1800 meters (Chute et al., 2008; Haefner and Musick, 1974; Steimle et al., 2001; Stevens and Guida, 2016; Weinberg and Keith, 2003). It has supported a small commercial fishery along the eastern U.S. (from the Gulf of Maine to Cape Hatteras, NC) since the early 1970s (Serchuk, 1977; Steimle et al., 2001; Wahle et al., 2008; Wigley et al., 1975). Currently, the *C. quinquedens* fishing industry restricts the harvest of adult males based on size (only individuals >94 mm carapace width are harvested; Chute et al., 2008), while females are protected from harvesting. However, data from a pair of studies suggests that recent fishing pressure on this species may have altered the size structure of populations, with observed declines in the size of harvested males (Wahle et al., 2008; Weinberg et al., 2003).

In 2002, the New England Fishery Management Council (NEFMC) implemented a fishery management plan (FMP) for *C. quinquedens*. The fishery for *C. quinquedens* is currently managed as a single stock divided into three fishing zones **(**NEFMC, 2016), based on prior studies and information about *C. quinquedens* population (demographic) structure, reproductive biology, movement, and connectivity (Chute et al., 2013; Diehl and Biesiot, 1994; Hines, 1988; Lux et al., 1982; Wahle et al., 2008; Weinberg et al., 2003; Weinberg and Keith, 2003)**.** A study of sequence variation at two loci in *C. quinquedens* populations from New England and the Gulf of Mexico showed unique (private) haplotypes in each population indicative of strong genetic subdivision and confirming the broad regional differences recognized for stocks of *C.*

quinquedens. However, the power to infer gene flow and measure genetic differentiation is limited with just two markers and the relatively small sample sizes employed in Weinberg et al. 2003 (n = 37). Additional study of genetic diversity in *C. quinquedens* populations using a larger number of higher resolution nuclear markers (i.e., microsatellites or single nucleotide polymorphisms) and with larger sample sizes is needed to address stock structure more adequately in this species.

The rapid progression in recent years of next-generation sequencing (NGS) technologies has provided researchers with lower-cost, more time-efficient methods to generate large amounts of genomic data, facilitating genome-wide analysis of genetic diversity and marker development in non-model marine species (Ekblom and Galindo, 2011; Koboldt et al., 2013). Microsatellites or simple sequence repeats (SSRs) are tandem repeats of nucleotides (1-6 bp repeats) throughout the genome of an organism. Microsatellites are highly abundant within the genome of all species, are highly polymorphic and informative, and are a very popular marker for researchers examining population structure, parentage, and the evolution of populations (Selkoe and Toonen, 2006). Next-generation sequencing of the genome or transcriptome in conjunction with bioinformatic analyses can be used to rapidly identify microsatellite markers in the genomes of virtually any species (Abdelkrim et al., 2009; Meglécz et al., 2010).

Recently, microsatellite markers have been used to establish the policies for fisheries management, to quantify population genetic diversity and population structure in species of decapod crustaceans (Abdul-Muneer, 2014; Maduna et al., 2014). For example, microsatellite markers have been used to study connectivity (gene flow) in several commercially important species e.g., *P. trituberculatus* (Xu and Liu, 2011), *C. opilio* (Puebla et al., 2003), *C. magister* (Toonen et al., 2004), *C. sapidus* (Steven et al. 2005), and *Scylla serrata* (Rumisha et al., 2017).

The addition of microsatellite loci to the currently available genetic markers for *C. quinquedens* will be helpful to study the stock structure and genetic diversity status of the species.

The main objective of this chapter is to use next-generation sequencing to develop microsatellite markers that can be used to assess the genetic diversity and population structure of *C. quinquedens* populations. As a federally managed fishery in the northeastern U.S., such markers are vital to understanding the population structure within its three management units. Here were generated 14 polymorphic microsatellite markers that can be used to examine genetic diversity parameters. In this chapter, we used three of the 14 markers in a preliminary examination of the genetic diversity of *C. quinquedens* with their management areas.

2. Materials and Methods

2.1 Collection of crabs

Forty-nine samples of *C. quinquedens* were collected in July 2014 during a cruise on the F/V Hannah Boden along the mid-Atlantic, off the coast of (Fig. 4.1 - fishing zone 2 & 3). Onboard the fishing vessel, a 200 µl hemolymph sample was collected from an individual animal in a 1:1 ratio (v:v) of hemolymph to anticoagulant and stored at 20°C. In July 2018, the Atlantic Red Crab Company (New Bedford, MA) collected female *C. quinquedens* from Hudson Canyon (Fig. 1 - fishing zone 1) and shipped live crabs (n = 49) to our laboratory at the Institute of Marine and Environmental Technology (Baltimore, MD). Hemolymph was extracted and stored using the same guidelines mentioned above. *C. quinquedens* samples were divided into three populations based on the three commercial fishing zones they were caught in (Fig. 4.1) (i) zone 1 - Hudson Canyon (HC), (ii) zone 2 - Mid-Atlantic Bight (MAB), and (iii) zone 3 - North Carolina (NC).

2.2 DNA extraction and Sequencing

DNA was extracted from hemocytes in a 200 µl sample of hemolymph using the High Pure PCR Template Preparation Kit (Roche) (Bembe et al., 2018). The extracted DNAs were quantified using a NanoDrop1000 spectrophotometer (FisherSci) and diluted with Tris-EDTA buffer to 1 ng/µl concentration. All six samples were sent to the University of Texas at Austin's Genomic Sequencing and Analysis Facility (UT-GSAF), where paired-end sequencing was done on the Illumina MiSeq platform 2x300 base pairs.

2.3 Microsatellite Detection via bioinformatics

Sequences were cleaned and assembled into contigs using the CLC Genomics Workbench (Qiagen). Contigs were then loaded into QDD 3.1 (Meglécz et al., 2014) software and ran using default parameters. In total, QDD identified the total perfect and imperfect microsatellites present, which were then narrowed down further by the following criteria. Microsatellites were additionally filtered to identify loci with primers that covered their flanking regions. Finally, QDD 3.1 identified microsatellites for which primer sets could be designed, based on the default criteria except for the flank_length which was set to 80 bp.

2.4 Microsatellite Primer Pair Selection

Three rounds of PCR testing were performed to narrow down the selection of microsatellite primer sets for downstream analysis. In the first round, all microsatellite primer sets designed with QDD 3.1 (n = 122) were tested using one ng DNA from the six sequenced individuals using the DNA from six adult red deep-sea crabs (n = 4 males and n = 2 females; plus a negative control) was amplified in 20 µl polymerase chain reactions (PCRs) and was prepared as follows: 2 µl genomic DNA template, 8 µl H_2O, 8 µl GoTaq 1.1x master mix, 1 µl

forward primer, and 1 µl reverse primer. Amplification conditions were as follows: initial denaturation 95°C for 5 mins then 28 cycles of denaturation for 95°C for 15 secs; annealing 53°C for 30 secs; extension 72°C for 1 min; a final extension of 72°C for 15 mins. PCR products were visualized on a 2% agarose gel for consistent amplification across all individuals. The second round (n = 59) used the same PCR conditions as round one. However, it used seven new individuals from the MAB region (plus a negative control). Primer pairs in round two were considered successful if one or two clear bands were present in at least 6 of the 7 samples on a 2% agarose gel. In round three (n = 25), markers were visualized again using gel electrophoresis and categorized as good (consistent amplification between 6 samples), okay (one or two clear bands in at least half of the samples), and poor (inconsistent amplification between samples, no detection of clear bands). Based on these initial marker tests, the initial 122 primer pairs were narrowed down to 14 that produced consistent amplification at the expected size ranges. New forward primers were then ordered containing a universal M13(-21) sequence attached to the 5' end of the primer (5′-TGTAAAACGACGGCCAGT-3′) and a complimentary 6-FAM™ fluorescently labeled M13 primer M13(-21) (Applied Biosystems) was also ordered following (Schuelke, 2000), for fragment analysis on the ABI 3130xl sequencer.

2.5 Fragment Analysis and Microsatellite Scoring

The 14 selected microsatellite primer pairs were further examined for consistent amplification and score ability during fragment analysis on the ABI 3130 xl. The genomic DNAs from *C. quinquedens* from three populations were tested - Hudson Canyon (HC; n = 49), Mid-Atlantic Bight (MAB; n = 31), and North Carolina (NC; n = 8). A multistep PCR assay adapted from Bembe et al., (2018) was used for fragment analysis. Briefly, at step one, PCR reactions

consisted of 2 µl H_2O, 2 µl template DNA at 5ng/µl, 5 µl GoTaq 1.1x master mix, 1 µl of each forward (tailed) primer at 5 µM/µl, and reverse primer at 5 µM using the following amplification conditions: initial denaturation 95°C for 5 mins then 35 cycles of denaturation for 95°C for 15 secs; annealing 57°C for 30 secs; extension 72°C for 1 min; a final extension of 72°C for 15 mins.

At step two, PCR reactions consisted of 4 µl H_2O, 2 µl template DNA at 5ng/µl, 5 µl GoTaq 1.1x master mix, 1 µl of each reverse and 6-FAM™ M13(-21) primer at 5 µM/µl using the following amplification conditions: initial denaturation 95°C for 5 mins then 10 cycles of denaturation for 95°C for 15 secs; annealing 53°C for 30 secs; extension 72°C for 1 min; a final extension of 72°C for 15 mins.

Six microliters of the PCR product from step 2 were added to 96 well-plates containing 9 µl Hi-Di™ formamide and 0.5 µl GeneScan™-500 LIZ® Size Standard (Applied Biosystems). Each sample plate was then spun down for one minute at 1000 rpm. Samples were denatured at 95°C for 3 minutes and then snap-cooled on ice for 2 – 5 minutes. Finally, plates were loaded into the 3130xl genetic analyzer (Applied Biosystems) for capillary electrophoresis (IMET, BASlab).

2.6 Genetic diversity assessment

Fragment analysis files from three loci Chq3, Chq9, and Chq11 of all samples (n = 264) were analyzed using Geneious Prime 2.9 (Kearse et al., 2012). The microsatellite plug-in for Geneious Prime was used to score alleles with all peaks scored manually. GenAlEx 6.5 (Peakall and Smouse, 2012) was used to calculate basic population diversity parameters: number of

alleles (N), null alleles (Na), observed heterozygosity (Ho), expected heterozygosity (H_E), fixation index (Fst), and Hardy-Weinberg Equilibrium (HWE).

3. Results

3.1 Sequencing results

The Illumina MiSeq sequencing produced 14,244,276 paired reads from the seven *C. quinquedens* genomic DNA samples. CLC Genomics Workbench produced 263,435 contigs (>80 bp). Contigs were analyzed in QDD, where 37,702 were identified as containing microsatellites repeats. Further filtering revealed 2,204 contigs contained perfect microsatellite repeats. A range of repeat motifs was identified. Di-, tri-, tetra-, penta- and hexanucleotide motifs were present at 4%, 10%, 21%, 25%, and 18%, respectively (Fig. 3.2).

3.2 Development of Microsatellite Primers

A total of 122 microsatellite primer pairs were developed from the QDD 3.1 program. Primer pairs were narrowed down through a series of PCR reactions initially evaluated with DNA samples of seven individuals if they showed polymorphism content. Following the first selection round, 59 primer pairs were chosen. In the second round, 25 primer pairs were selected for testing. Of these, 14 microsatellite markers were chosen based on their consistent amplification among samples and the presence of one or two clear bands visualized on an agarose gel. The final primers were selected, including three (from 14) pairs two tri-, and one tetranucleotide motifs, and were examined in 88 individuals.

3.4 Genetic Diversity

To provide an initial assessment of genetic diversity in deep-sea reds crabs with the newly developed microsatellite markers, three of the primer pairs (Chq3, Chq9, and Chq11) were

genotyped from the three populations (management area): HC (n = 49), MAB (n = 31), and NC (n = 8; Fig. 4.1). Across the three loci mean expected (H_e) and observed heterozygosity (H_o) were 0.879 and 1, respectively, thus indicating high levels of genetic diversity for these three populations. Across the three loci, the number of alleles varied from 8 to 19 (Table 6). Pairwise Fst estimates were calculated to determine genetic differentiation among the three populations. The smallest F_{ST} value was between the HC and MAB populations (F_{ST} = 0.013) and the largest was observed between MAB and NC (0.035) (Table 7). Despite the relatively low genetic differentiation among all the populations, low gene differentiation between HC and MAB indicates gene flow amongst the populations within the *C. quinquedens* management area (Table 8). However, the higher FST we observed between MAB and NC begins to present the potential barrier where the subdivision between *C. quinquedens* populations.

4. Discussion

This chapter used sequences produced from NGS technology combined with a bioinformatics workflow to successfully develop the first microsatellite markers for the red deep-sea crab *C. quinquedens.* A preliminary population genetic analysis was then carried out using three loci to examine *C. quinquedens* genetic diversity and connectivity within its management area. An increasing number of microsatellite marker studies take advantage of the power of NGS technology to produce large amounts of sequencing data. NGS sequencing and the subsequent bioinformatic analysis enabled the development of over a hundred microsatellite markers in a short period at a relatively low cost.

Before this study, the only markers that existed for examining the population genetics of *C. quinquedens* were developed over a decade ago (Diehl and Biesiot, 1994; Weinberg et al., 2003) and were not specific to the deep-sea red crab. Markers for 14 microsatellite loci were

successfully developed and can be used to assess genetic diversity in *C. quinquedens*. The repeat motif composition of the markers includes di- (6), tri- (4), and tetra- (4). These are the first microsatellite loci developed for the genus *Chaceon* and, with further testing, should be useful for future analyses of population genetics in *C. quinquedens* and possibly in other members of the genus.

Three microsatellite markers were used to assess basic population genetic parameters for 88 crabs from across the three management areas, Chq3, Chq9, and the Chq11. Observed and expected heterozygosity was high, as is typically observed for marine species (Hornick and Plough, 2019; Plough et al., 2014). Two loci showed significant deviation from HWE for HC and MAB sites (Chq3 and Chq9), which is indicative of null alleles, which is common in microsatellite markers. The smallest F_{ST} value was between the HC and MAB populations (F_{ST} = 0.013), and the largest was observed between MAB and NC (F_{ST} = 0.035).

Both studies by Diehl and Biesiot (1994) and Weinberg et al. (2003) showed that genetic differentiation becomes apparent when examining populations from New England and the Florida Coast and the Gulf of Mexico. The results from our microsatellite analysis show that although significant gene flow is likely occurring between all populations, comparisons between more distant populations produce larger values of F_{ST}, possibly consistent with a pattern of isolation by distance. In the future, it will be important to extend the sampling to more populations along the U.S. Atlantic coast to determine if the same patterns of genetic differentiation exist. To this end, the current study planned to incorporate the samples from the Florida border and/or the Gulf of Mexico. However, our attempts to collect the samples in this region were unsuccessful.

Movement in the red deep-sea crab is an important factor to consider when understanding genetic diversity. Migration or movement of individuals between populations is often a key driver of genetic exchange among populations, and relatively few migrants per generation are needed to completely homogenize allele frequencies in the marine environment (Meirmans and Hedrick, 2011). Like other deep-sea crabs, *C. quinquedens* have been documented at several life stages moving up and down-slope (Haefner and Musick, 1974; Stevens and Guida, 2016; Wahle et al., 2008; Weinberg and Keith, 2005). However, unlike the constant movement up/downslope tagging studies, deep-sea red crabs make limited movement horizontally along the slope (Lux et al., 1982).

5. Conclusions

In this study, the first microsatellite loci for the deep-sea red crab were isolated and tested, yielding 14 high-quality candidates, of which three were examined extensively for an initial assessment of diversity and population structure in this species. These loci will be important in future studies examining the genetic diversity of *C. quinquedens*, especially those looking to assess the geographic limits of the current stocks in fishing zones. In the US, the red deep-sea crab population extends south into the Gulf of Mexico. Additional studies are needed to update our understanding of the genetic structure of *C. quinquedens* across its entire range, and the newly developed microsatellite markers developed here will be key to this effort.

References

Abdelkrim, J., Robertson, B.C., Stanton, J.-A.L., Gemmell, N.J., 2009. Fast, cost-effective development of species-specific microsatellite markers by genomic sequencing. BioTechniques 46, 185–192. https://doi.org/10.2144/000113084

Abdul-Muneer, P.M., 2014. Application of Microsatellite Markers in Conservation Genetics and Fisheries Management: Recent Advances in Population Structure Analysis and Conservation Strategies. Genetics Research International 2014, 1–11. https://doi.org/10.1155/2014/691759

Bembe, S., Zmora, N., Williams, E., Place, A.R., Liang, D., Chung, J.S., 2018. Effects of temperature and photoperiod on hemolymph vitellogenin levels during spawning events of the blue crab, *Callinectes sapidus*, in captivity. Aquaculture Research 49, 2201–2209. https://doi.org/10.1111/are.13676

Chute, A., Jacobson, L., Rago, P., MacCall, A., 2008. Deep Sea red crab. Northeast Data Poor Stocks Working Group Meeting 1–18.

Chute, A., Jacobson, L., Redding, B., 2013. Northeast Fisheries Science Center Clarification of Methods Used to Determine Deep Sea Red Crab (Chaceon quinquedens) Abundance from Data Collected during the 1974 Photographic Survey.

Diehl, W.J., Biesiot, P.M., 1994. Relationships between multilocus heterozygosity and morphometric indices in a population of the deep-sea red crab *Chaceon quinquedens* (Smith). Journal of Experimental Marine Biology and Ecology 182, 237–250. https://doi.org/10.1016/0022-0981(94)90054-X

Ekblom, R., Galindo, J., 2011. Applications of next-generation sequencing in molecular ecology of non-model organisms. Heredity 107, 1–15. https://doi.org/10.1038/hdy.2010.152

Haefner, P.A., Musick, J.A., 1974. Observations on distribution and abundance of red crabs in Norfolk Canyon and adjacent continental slope. Marine Fisheries Review 36, 31–34.

Hines, A.H., 1988. Fecundity and Reproductive Output in Two Species of Deep-Sea Crabs, Geryon fenneri and G. quinquedens (Decapoda: Brachyura), Source: Journal of Crustacean Biology.

Hornick, K.M., Plough, L. v., 2019. Tracking genetic diversity in a large-scale oyster restoration program: effects of hatchery propagation and initial characterization of diversity on restored vs. wild reefs. Heredity 123, 92–105. https://doi.org/10.1038/s41437-019-0202-6

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Meintjes, P., Drummond, A., 2012. Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28, 1647–1649. https://doi.org/10.1093/bioinformatics/bts199

Koboldt, D.C., Steinberg, K.M., Larson, D.E., Wilson, R.K., Mardis, E.R., 2013. The next-generation sequencing revolution and its impact on genomics. Cell 155, 27–38. https://doi.org/10.1016/j.cell.2013.09.006

Lux, F.E., Ganz, A.R., Rathjen, W.F., 1982. Marking studies on the red crab *Geryon quinquedens* Smith off southern New England. Journal of Shellfish Research 2, 71–80.

Maduna, S.N., Rossouw, C., Roodt-Wilding, R., Bester-van der Merwe, A.E., 2014. Microsatellite cross-species amplification and utility in southern African elasmobranchs: A valuable resource for fisheries management and conservation. BMC Research Notes 7, 352. https://doi.org/10.1186/1756-0500-7-352

Meglécz, E., Costedoat, C., Dubut, V., Gilles, A., Malausa, T., Pech, N., Martin, J.-F., 2010. QDD: a user-friendly program to select microsatellite markers and design primers from large sequencing projects. Bioinformatics (Oxford, England) 26, 403–4. https://doi.org/10.1093/bioinformatics/btp670

Meglécz, E., Pech, N., Gilles, A., Dubut, V., Hingamp, P., Trilles, A., Grenier, R., Martin, J.-F., 2014. QDD version 3.1: a user-friendly computer program for microsatellite selection and primer design revisited: experimental validation of variables determining genotyping success rate. Molecular Ecology Resources 14, 1302–1313. https://doi.org/10.1111/1755-0998.12271

Meirmans, P.G., Hedrick, P.W., 2011. Assessing population structure: F_{ST} and related measures. Molecular Ecology Resources 11, 5–18. https://doi.org/10.1111/j.1755-0998.2010.02927.x

Peakall, R., Smouse, P.E., 2012. GenAlEx 6.5: genetic analysis in Excel. Population genetic software for teaching and research--an update. Bioinformatics (Oxford, England) 28, 2537–9. https://doi.org/10.1093/bioinformatics/bts460

Plough, L. v., Moran, A., Marko, P., 2014. Density drives polyandry and relatedness influences paternal success in the Pacific gooseneck barnacle, Pollicipes elegans. BMC Evolutionary Biology 14. https://doi.org/10.1186/1471-2148-14-81

Puebla, O., Parent, É., Sévigny, J.M., 2003. New microsatellite markers for the snow crab Chionoecetes opilio (Brachyura: Majidae). Molecular Ecology Notes 3, 644–646. https://doi.org/10.1046/j.1471-8286.2003.00542.x

Rumisha, C., Huyghe, F., Rapanoel, D., Mascaux, N., Kochzius, M., 2017. Genetic diversity and connectivity in the East African giant mud crab *Scylla serrata*: Implications for fisheries management. PLoS ONE 12. https://doi.org/10.1371/journal.pone.0186817

Schuelke, M., 2000. An economic method for the fluorescent labeling of PCR fragments. Nature Biotechnology 18, 233–234.

Selkoe, K.A., Toonen, R.J., 2006. Microsatellites for ecologists: A practical guide to using and evaluating microsatellite markers. Ecology Letters. https://doi.org/10.1111/j.1461-0248.2006.00889.x

Serchuk, F.M., 1977. Assessment of red crab (*Geryon quinquedens*) populations in the northwest Atlantic. Northeast Fisheries Science Center Ref. Doc. 77, 1–15.

Steimle, F.W., Zetlin, C.A., Chang, S., 2001. Red deepsea crab, *Chaceon (Geryon) quinquedens*, life history and habitat characteristics., NOAA Tech. Memo. NMFS-NE-163.

Stevens, B.G., Guida, V., 2016. Depth and temperature distribution, morphometrics, and sex ratios of red deepsea crab (*Chaceon quinquedens*) at 4 sampling sites in the Mid-Atlantic Bight. Fishery Bulletin 114, 343–359. https://doi.org/10.7755/FB.114.3.7

Toonen, R.J., Locke, M., Grosberg, R., 2004. Isolation and characterization of polymorphic microsatellite loci from the Dungeness crab Cancer magister. Molecular Ecology Notes 4, 30–32. https://doi.org/10.1046/j.1471-8286.2003.00562.x

Wahle, R.A., Bergeron, C.E., Chute, A.S., Jacobson, L.D., Chen, Y., 2008. The Northwest Atlantic deep-sea red crab (*Chaceon quinquedens*) population before and after the onset of harvesting. ICES Journal of Marine Science 65, 862–872.

Weinberg, J., Keith, C., 2005. Revisiting population size-structure of the deep-sea red crab, *Chaceon quinquedens*. Crustaceana 78, 245–246. https://doi.org/10.1163/1568540054020604

Weinberg, J.R., Dahlgren, T.G., Trowbridge, N., Halanych, K.M., 2003. Genetic differences within and between species of deep-sea crabs (*Chaceon*) from the North Atlantic Ocean. Biological Bulletin 204, 318–326.

Weinberg, J.R., Keith, C., 2003. Population size-structure of harvested deep-sea red crabs (*Chaceon quinquedens*) in the northwest Atlantic Ocean. Crustaceana 79, 819–833.

Wigley, R.L., Theroux, R.B., Murray, H.E., 1975. Deep-sea red crab, *Geryon quinquedens*, survey off northeastern United States. Marine Fisheries Review 1154, 1–22.

Xu, Q., Liu, R., 2011. Development and characterization of microsatellite markers for genetic analysis of the swimming crab, *Portunus trituberculatus*. Biochemical Genetics 49, 202–212. https://doi.org/10.1007/s10528-010-9399-z

Table 6. Characteristics of 14 microsatellite markers analyzed in 88 red deep-sea crabs (*C. quinquedens).*

Locus	Motif	Size (bp)
Chq2	ACAG	282
Chq3	AAAT	263
Chq5	ACTC	246
Chq7	ACGC	110
Chq8	ATC	341
Chq9	AGG	310
Chq11	ACT	282
Chq16	AT	184
Chq17	AT	262
Chq18	AT	210
Chq20	AC	360
Chq21	AC	270
Chq23	AG	310
Chq24	AG	263

Table 7. Characterization of 3 polymorphic microsatellite loci for the red deep-sea crab, *C. quinquedens.* Samples genotyped (N), number of alleles (N_a), number of effective alleles (N_e), Shannon's Information Index (I), observed heterozygosity (H_o), expected heterozygosity (H_E), and fixation index (Fst).

	N	N_a	N_e	I	H_o	H_E	Fst
Chq3	15.667	8.667	6.060	1.931	1.000	0.828	0.032
Chq9	26.333	19.000	12.597	2.649	1.000	0.910	0.026
Chq11	21.000	17.333	11.601	2.516	1.000	0.899	0.036

Table 8. Characterization of 3 polymorphic microsatellite loci for the red deep-sea crab, *C. quinquedens* by locus and site. Samples genotyped (N), number of alleles (N_a), number of effective alleles (N_e), Shannon's Information Index (I), observed heterozygosity (H_o), expected heterozygosity (H_E), and Hardy-Weinberg Equilibrium (HWE).

Pop	Locus	N	N_a	N_e	I	H_o	H_E	HWE
HC	Chq3	30	10	6.569	2.032	1.000	0.848	0.000^{***}
	Chq9	45	28	18.000	3.087	1.000	0.944	0.263
	Chq11	45	30	16.875	3.077	1.000	0.941	0.000^{***}
MAB	Chq3	9	7	4.500	1.692	1.000	0.778	0.026^{*}
	Chq9	27	19	12.252	2.692	1.000	0.918	0.003^{**}
	Chq11	14	15	11.529	2.564	1.000	0.913	0.777

NC	Chq3	8	9	7.111	2.068	1.000	0.859	0.369
	Chq9	7	10	7.538	2.168	1.000	0.867	0.858
	Chq11	4	7	6.400	1.906	1.000	0.844	0.521

Statistical significance is noted at *0.05, **0.01, and ***0.001.

Table 9. The analysis of Fst values for comparisons of the three *C. quinquedens* populations using analysis of molecular variance (AMOVA).

	HC	**MAB**	**NC**
HC	0.000		
MAB	0.013	0.000	
NC	0.023	0.035	0.000

Figures:

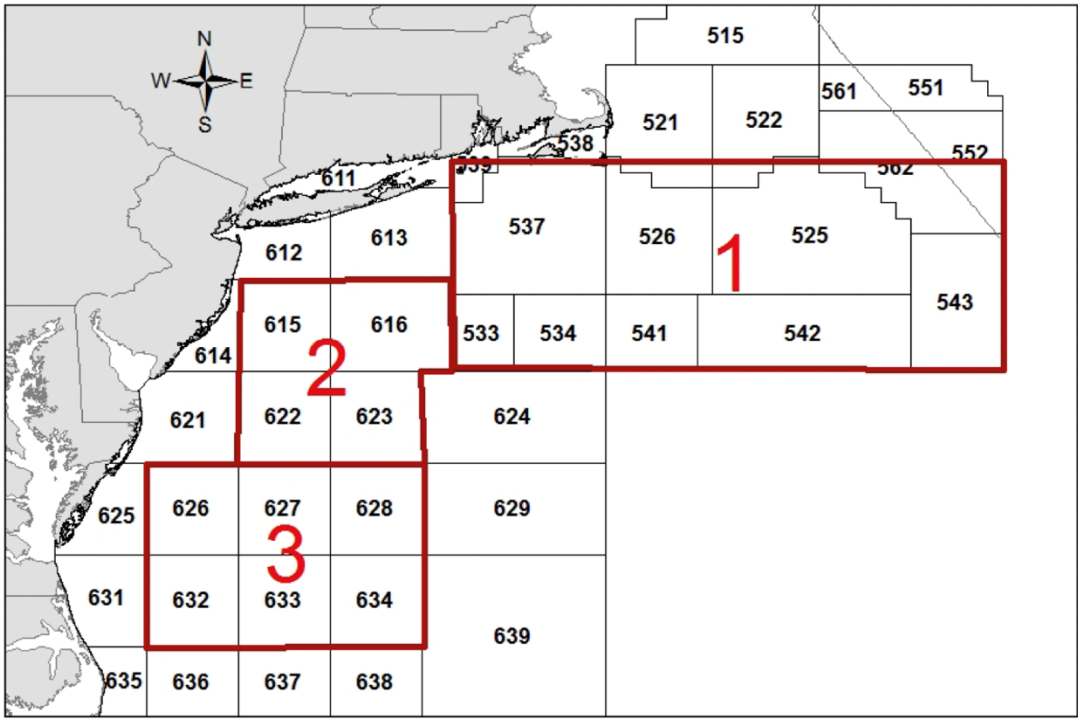


Fig. 4.1. Map showing the National Marine Fishery Service (NMFS) statistical areas, and the *C. quinquedens* fishing zones 1-3**.**

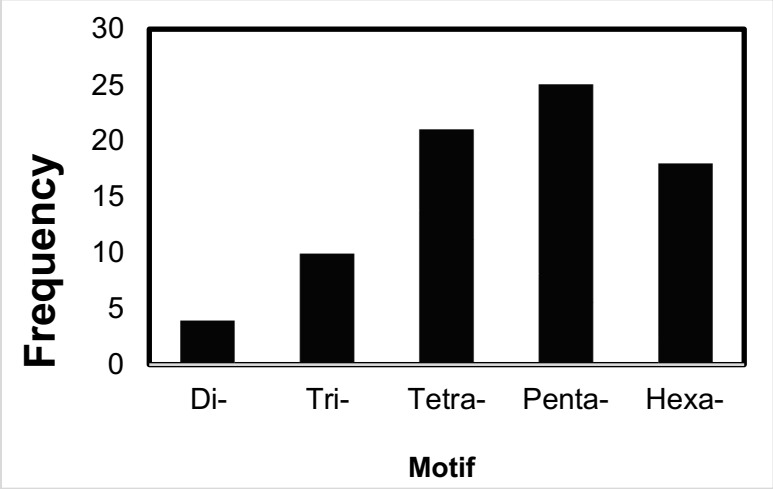


Fig. 4.2. Frequency of microsatellite repeat motif types in *C. quinquedens***.**

Chapter 5: General Discussion and Future Directions

This dissertation investigated the reproductive physiology of female red deep-sea crabs, *Chaceon quinquedens* by elucidating hormonal regulators produced in the eyestalk ganglia and hepatopancreas tissues. In chapter 2, this study reports the first description of neuropeptides produced in the eyestalk ganglia of female *C. quinquedens*. The assembly contained 28 unique eyestalk-produced neuropeptides, of which seven may have a role in vitellogenesis in this species.

Traditional molecular cloning methods were used to characterize the protein sequence of the crustacean hyperglycemic hormone (CHH) and the molt-inhibiting hormone (MIH). These pleiotropic hormones are involved in many physiological activities in crustaceans (Hsu et al., 2006; Reddy and Reddy, 2006), and our work aimed to investigate their role in female reproduction. Both MIH and CHH are traditionally known to display molt-inhibiting activity and may play an important role in the vitellogenesis of females in species such as *C. quinquedens*.

CHH expression did not differ significantly between ovarian stages which indicate that the continuous production and release are important to both molting and vitellogenesis. The expression of MIH during ovarian development exhibited a similar trend to that of vitellogenin. Increased expression of MIH keeps females in the intermolt phase indicates the importance of the connection to vitellogenesis. During these long molt cycles, females can direct all the necessary energy to reproduction. A biennial reproductive cycle was proposed (Martínez-Rivera et al., 2020; Stevens and Guida, 2016). With an intermolt phase lasting so long, adult females could undergo at least 3 separate vitellogenesis events within a single molt cycle.

In *C. quinquedens*, it is assumed that vitellogenesis is a continuous process throughout adult life therefore it is vital to understand the process in females. In chapter 3, a hepatopancreas transcriptome is assembled from females at early and mid-vitellogenesis (stage 1 and 3,

respectively) and was used to identify the 2,570 aa *C. quinquedens* vitellogenin protein. This is the also first reported complete vitellogenin sequence of a deep-sea decapod.

In crustaceans, vitellogenin must be post-translationally modified prior to becoming the major egg yolk protein vitellin (Zmora et al., 2007). The large vitellogenin protein is first cleaved in the hepatopancreas, released into the hemolymph where it is transported, and then absorbed into the ovary. Subtilisin-like endopeptidases are speculated to be responsible for this initial cut. The hepatopancreas transcriptome was searched to discover the presence of potential candidates with the potential to conduct this first cleavage of vitellogenin. Three enzymes were identified furin, a trypsin-like serine protease, and proprotein convertase subtilisin/Kexin type 7. Trypsin-like serine protease and proprotein convertase subtilisin/Kexin type 7 values increase between stages 1 and 3, which is the same trend for vitellogenin. The increase in values between each of these enzymes could be responsible for the cleavage of increased vitellogenin protein during stage 3.

In chapter 4, the first microsatellite markers for *C. quinquedens* were developed. Fourteen high-quality microsatellite loci were generated and can be used to assess the genetic diversity of *C. quinquedens* populations in the US. The *C. quinquedens* fishery predominantly occurs from the Gulf of Maine down to Cape Hatteras (Lindberg and Wenner, 1990; Steimle et al., 2001). Previously only two studies explored the genetic diversity of *C. quinquedens* (Diehl and Biesiot, 1994; Weinberg et al., 2003). These markers are important tools needed by the fishery to gain a recent view of the population structure for this species within their management range.

The fishery for *C. quinquedens* is currently managed as a single stock divided into three fishing zones (Chute et al., 2013; Steimle et al., 2001). Three of the 14 loci were used to assess basic genetic diversity statistics in order to test the functionality of these polymorphic markers.

The average expected heterozygosity was 0.879, revealing a high level of genetic diversity between the populations. Hardy-Weinberg Equilibrium was significantly different for the loci Chq3 in the Hudson Canyon and mid-Atlantic bight populations. A study of sequence variation at two loci in *C. quinquedens* populations from New England and the Gulf of Mexico showed unique (private) haplotypes in each population indicative of strong genetic subdivision and confirming the broad regional differences recognized for stocks of *C. quinquedens* (Weinberg et al., 2003).

Populations of *C. quinquedens* from the mid-Atlantic bight and North Carolina had the highest Fst (0.035) of the observed sites. This result shows that as we enter the southern portion of their range genetic subdivision is beginning to increase. It is important to expand sampling to other areas along the population's distribution. This result hints that as the early studies on the population genetics of this species, there may be higher levels of genetic diversity and differentiation (Diehl and Biesiot, 1994; Weinberg et al., 2003).

Future Work

The eyestalk and hepatopancreas transcriptomes produced by this research provide the first look into regulators of reproduction in females. Transcriptomes produced can be used to investigate many factors participating in or influencing other important physiological processes. The neuropeptides identified in the eyestalk transcriptome can provide a better understanding of the long molt cycle in this species. Levels of these molting-related peptides can be examined by the ovarian stage, which can further elucidate the connection between the two.

The crustacean hepatopancreas is a digestive tissue that produces many proteins important for processes such as growth, reproduction, and nutrition. In *C. quinquedens* the hepatopancreas assembly can be used to investigate other vitellogenesis-related transcripts and their expression

profiles in females at ovarian stages 1 and 3. The hepatopancreas transcriptome produced the full-length protein sequence for vitellogenin and identified candidate cleavage enzymes. Additional studies can be done to further investigate the relationship between subtilisin-like endopeptidase and vitellogenin in this species.

In future studies, hemolymph titers of MIH and CHH need to be determined throughout ovarian development to define further their role in vitellogenesis and reproduction in this species. Vitellogenin being transported through the hemolymph to the ovary can be used as a marker to further define where reproductively a population of *C. quinquedens* females are. Understanding where the populations of females can be an essential piece of information for managers of this species.

Now that the first set of nuclear markers for this species has been designed, they can be used to answer many other questions i.e., how many *C. quinquedens* stocks are there? How much gene flow occurs among these stocks? These markers will be a key component to researchers attempting to examine the deep-sea red crab population throughout their entire U.S. range (the Gulf of Maine into the Gulf of Mexico).

Another vital need for future studies is the ability to sample crabs at depths deeper than what the current fishery catches them. Juvenile *C. quinquedens* settle down at the deepest portions of the depth range (>800 m). This limits our ability to collect and study juvenile females, which presented a small gap in the expression profiles for VTG. Insight from individuals collected at the deepest depths is also important to the study of molt frequency in this species.

General Conclusion

This dissertation is a compliment and a contribution to knowledge and understanding of the reproductive physiology of red deep-sea crab females. Members of the crustacean hyperglycemic hormone family identified in this study are very similar to sequences found in other decapods and appear to also be pleiotropic in nature. Similarly, the same is true for the vitellogenin sequence identified, it contained many of the same features and domains as other crab species (Jimenez-Gutierrez et al., 2019; Thongda et al., 2015). Investigating these hormones throughout ovarian development in this species is getting us closer to having a baseline tool that can be incorporated into studies examining the stock. Fisheries managers can rely on a measurement of any of these hormones to detect the reproductive status of the female population. The information gained from reproduction and population genetics is both two great tools to provide an abundance of biological data to guide decision-making. A sample of hemolymph from an individual crab can provide researchers with information on the population structure thanks to the newly developed microsatellite markers. Most importantly, assays developed to obtain these measurements from hemolymph samples will provide important information without sacrificing the animal.

References

Chute, A., Jacobson, L., Redding, B., 2013. Northeast Fisheries Science Center Clarification of Methods Used to Determine Deep Sea Red Crab (Chaceon quinquedens) Abundance from Data Collected during the 1974 Photographic Survey.

Diehl, W.J., Biesiot, P.M., 1994. Relationships between multilocus heterozygosity and morphometric indices in a population of the deep-sea red crab *Chaceon quinquedens* (Smith). Journal of Experimental Marine Biology and Ecology 182, 237–250. https://doi.org/10.1016/0022-0981(94)90054-X

Hsu, Y.-W. a, Messinger, D.I., Chung, J.S., Webster, S.G., de la Iglesia, H.O., Christie, A.E., 2006. Members of the crustacean hyperglycemic hormone (CHH) peptide family are differentially distributed both between and within the neuroendocrine organs of *Cancer* crabs: implications for differential release and pleiotropic function. The Journal of experimental biology 209, 3241–56. https://doi.org/10.1242/jeb.02372

Jimenez-Gutierrez, S., Cadena-Caballero, C.E., Barrios-Hernandez, C., Perez-Gonzalez, R., Martinez-Perez, F., Jimenez-Gutierrez, L.R., 2019. Crustacean vitellogenin: A systematic and experimental analysis of their genes, genomes, mRNAs and proteins; and perspective to Next Generation Sequencing. Crustaceana 92, 1169–1205. https://doi.org/10.1163/15685403-00003930

Lindberg, W.J., Wenner, E.L., 1990. Geryonid Crabs and associated continental slope fauna: A research workshop report. NOAA National Undersea Research Center 58.

Martínez-Rivera, S., Long, W.C., Stevens, B.G., 2020. Physiological and behavioral sexual maturity of female red deep-sea crabs *Chaceon quinquedens* (Smith, 1879) (Decapoda: Brachyura: Geryonidae) in the Mid-Atlantic Bight. Journal of Crustacean Biology 40, 330–340. https://doi.org/10.1093/JCBIOL/RUAA007

Reddy, P.R., Reddy, P.S., 2006. Isolation of peptide hormones with pleiotropic activities in the freshwater crab, *Oziotelphusa senex senex*. Aquaculture 259, 424–431. https://doi.org/10.1016/j.aquaculture.2006.05.019

Steimle, F.W., Zetlin, C.A., Chang, S., 2001. Red deepsea crab, *Chaceon (Geryon) quinquedens*, life history and habitat characteristics., NOAA Tech. Memo. NMFS-NE-163.

Stevens, B.G., Guida, V., 2016. Depth and temperature distribution, morphometrics, and sex ratios of red deepsea crab (*Chaceon quinquedens*) at 4 sampling sites in the Mid-Atlantic Bight. Fishery Bulletin 114, 343–359. https://doi.org/10.7755/FB.114.3.7

Thongda, W., Chung, J.S., Tsutsui, N., Zmora, N., Katenta, A., 2015. Seasonal variations in reproductive activity of the blue crab, *Callinectes sapidus*: Vitellogenin expression and levels of vitellogenin in the hemolymph during ovarian development. Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology 179, 35–43. https://doi.org/10.1016/j.cbpa.2014.08.019

Weinberg, J.R., Dahlgren, T.G., Trowbridge, N., Halanych, K.M., 2003. Genetic differences within and between species of deep-sea crabs (*Chaceon*) from the North Atlantic Ocean. Biological Bulletin 204, 318–326.

Zmora, N., Trant, J., Chan, S.M., Chung, J.S., 2007. Vitellogenin and its messenger RNA during ovarian development in the female blue crab, *Callinectes sapidus*: gene expression, synthesis, transport, and cleavage. Biology of Reproduction 77, 138–146. https://doi.org/10.1095/biolreprod.106.055483